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MICROSCOPIC AND BIOCHEMICAL STUDIES ON A CHLOROPHYLL MUTANT OF GATEWAY BARLEY

by

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ABSTRACT

The pigment content, chloroplast structure and nitrogen metabolism of a viable chlorophyll mutant of Gateway barley were examined. The following are the principle findings and conclusions.

1. The Gateway mutant is a "viriscens" type of viridis or pale green chlorophyll mutant, able to recover greenness with age.

2. Young mutant seedlings are low in all chloroplast pigments, and the plastids are small and irregular in shape, containing large vesicles but no normal lamellae or grana.

3. Acid-soluble nitrogen and free amino acid levels are very high in the young mutant, and the relative balance of amino acids is distorted. Free ribonucleotide levels are low.

4. The concentration of free serine in young mutant seedlings is extraordinarily high, which may indicate that this amino acid has an important role in chloroplast development, possibly in its capacity as a constituent of phospholipid in membranes and lamellar structures.

5. Low pigment levels and aberrations in chloroplast ultrastructure in the young mutant could arise from an abnormality in the synthesis of lipoprotein.

6. The recovery of greenness with age in the mutant is accompanied by recovery of normal chloroplast size and structure, and nearly normal nitrogen levels, free amino acid balance and free ribonucleotide concentrations.

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TABLE OF CONTENTS

TABLE OF CONTENTS - Con'd.

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES - Con't.

INTRODUCTION

The accumulation of chlorophyll in plants has been shown to be associated with the integration of pigment into highly organized lipoprotein structures. Three approaches have been taken towards elucidating the details of this process. Estimations of pigments during the greening of intact tissues have provided data relevant to the mechanism of chlorophyll biosynthesis. Electron microscopy has revealed the ultrastructure of the chloroplast and the interdependence of pigment accumulation and chloroplast development. Biophysical and biochemical investigations of chlorophyll *in vivo* have demonstrated the attachment of pigment to a lipoprotein carrier and its consequent stabilization.

In studies of pigment biosynthesis and chloroplast ultrastructure useful information has been obtained from plants in which mutations have occurred in the chlorophyll-forming apparatus, particularly from the albino and yellow types of mutant. Biochemical investigations of these mutants have been limited, to date, though they might well provide some understanding of the nature of the pigment carrier and the chemical processes involved in chloroplast differentiation.

The pale green mutant of Gateway barley used in the present studies is of special interest because of its ability to recover with age from the effects of the mutation. It thereby provides the

opportunity for examining the abnormalities associated with the mutation and the changes which take place during recovery. Accordingly, the purpose of this investigation was twofold: first to determine the alterations in pigmentation and chloroplast structure caused by the mutation, and the extent to which these were eventually restored; and second to try to find a chemical basis for these alterations by examining changes in the metabolism of related cell constituents.

LITERATURE REVIEW

1. The Nature and Biosynthesis of the Chlorophyll Molecule.

The chlorophyll molecule consists essentially of a hydrophilic porphyrin nucleus complexed with an atom of magnesium and esterified with the lipophilic long-chain alcohol, phytol. When chlorophyll is distributed at an oil-water interface, the molecules arrange themselves in a monolayer with their porphyrin "heads" in water and their phytol "tails" in oil. This property of the molecule is thought to determine its distribution within the chloroplast, the phytol group acting as an anchor for the light-absorbing porphyrin nucleus.

In higher plants two forms of chlorophyll are found, designated as chlorophyll a and chlorophyll b. They differ slightly in chemical composition, and absorption spectra. Chlorophyll a has its major absorption peaks in acetone at 663 and 430 mμ and chlorophyll b at 645 and 455 mμ (MacKinney, 1940). In most higher plants they are found in the ratio of approximately three molecules of chlorophyll a to one of chlorophyll b.

The pathway of chlorophyll biosynthesis is similar in its early stages to that of heme biosynthesis in animals (Granick, 1951; Gibson, et al., 1961). It involves the condensation of glycine with succinyl-coenzyme A to give delta-aminolevulinic acid which is then converted to the pyrrole compound, porphobilinogen. Four molecules

of porphobilinogen combine to form the porphyrin compound, protoporphyrin-9, common to both plants and animals. The reaction sequence by which protoporphyrin-9 is converted to protochlorophyll, the immediate precursor of chlorophyll, is not clearly understood. Granick (1951) proposed a scheme based on the presence of certain uroporphyrins and coproporphyrins in chlorophyll mutants of Chlorella. This scheme has been criticised, however (Rabinowitch, 1951; Gibson, et al., 1961), on the grounds that the presence of certain porphyrin compounds in an organism whose chlorophyll-synthesising mechanism has been blocked does not necessarily mean that these compounds are intermediates in chlorophyll formation.

The conversion of protochlorophyll to chlorophyll a has been studied in great detail by many investigators. This reaction requires light in most plants. (Certain algae and gymnosperms are exceptions.) The presence of protochlorophyll in green tissues is difficult to detect because its concentration is extremely low. It has been isolated from etiolated tissues, however, where it is able to accumulate to a limited extent, and also from the inner seed coats of squash (Smith and Young, 1956). It differs from chlorophyll a chemically only in having two hydrogen atoms less (i.e. one extra double bond) on one of the pyrrole rings. Its absorption spectrum is also different, the major peaks in acetone being at 623 and 432 mμ (Koski and Smith, 1948).

That protochlorophyll is in fact the precursor of chlorophyll a was established by Koski (1950) who showed that the rapid formation of chlorophyll a in etiolated corn seedlings on exposure to light is accompanied by an exactly equivalent decrease in protochlorophyll. It has also been shown that protochlorophyll is the active light absorber for its own conversion to chlorophyll a (Koski, et al., 1951).

Koski (1950) has followed chlorophyll formation during prolonged exposure of etiolated seedlings to light. After the initial rapid conversion of protochlorophyll to chlorophyll a there is a lag period of about an hour during which no additional chlorophyll is formed, presumably because more precursor must first be synthesised. At the end of an hour the chlorophyll a concentration begins to rise, and at the same time chlorophyll b is first detectable. This is the second phase of chlorophyll formation. It has been proposed that chlorophyll b is derived from chlorophyll a since it appears later but both chlorophylls could arise from a common precursor before protochlorophyll (Smith and Young, 1956). The amounts of the two chlorophylls rise slowly at first but at an accelerating rate which is suggestive of an autocatalytic process. Finally a stage is reached at which no further accumulation of chlorophyll occurs and the concentration remains constant.

The rate at which protochlorophyll is formed in darkness

has been studied by Smith and Young (1956) and by Virgin (1955). It appears that this rate is not rapid enough to account for the rate of chlorophyll formation in the second phase. This means either that protochlorophyll is formed more rapidly in the light than in the dark, or that chlorophyll formation takes place via another pathway during the second phase. That the first possibility is more probable is supported by the findings of Withrow, et al. (1956) and Wolf, et al. (1957). This group showed that the lag phase of chlorophyll formation is overcome by previous treatment with low energy light followed by several hours of darkness, and that protochlorophyll synthesis is stimulated by such treatment. Recently Virgin (1961) showed that when young green leaves are placed in darkness protochlorophyll is formed very rapidly at first, though this rate decreases with time. In older leaves which have stopped growing and whose chlorophyll content is neither increasing nor turning over (Perkins and Roberts, 1960), protochlorophyll is not formed to any extent during a 5 hour period of darkness. Thus the evidence favors the view that chlorophyll is always formed from protochlorophyll and not from any other precursor.

With respect to the question of when phytol is added to the porphyrin nucleus, Wolf and Price (1957) demonstrated that esterification occurs after photoconversion has taken place. Thus, in fact, the reaction involves conversion of protochloro-

phyllide (protochlorophyll minus phytol) to chlorophyllide a. The addition of phytol takes place within an hour after conversion. This has been confirmed by Virgin (1960) who pointed out that though the main part of the precursor present in etiolated leaves is "unphytolised," the small fraction which is esterified may correspond to the protochlorophyll which remains unchanged after irradiation (conversion is never complete).

Several facts suggest that chlorophyll is associated in the chloroplast with a proteinaceous carrier (Kupke and French, 1960). It is more stable in the leaf than it is in organic solvents, i.e. it is more resistant to bleaching. The red absorption maxima of chlorophyll in vivo, and of water extracts of tissues and artificial chlorophyll-protein complexes, are shifted 10-15 mμ toward longer wavelengths compared with those of chlorophyll in organic solvents. Also, when protochlorophyll is extracted with organic solvents it cannot be converted by light to chlorophyll a, whereas if it is extracted together with protein in buffered aqueous solutions it can be converted (Smith, et al., 1956). Further evidence was provided by the spectroscopic studies of Shibata (1957) on chlorophyll formation in intact leaves. Using etiolated bean and corn leaves he followed changes in the absorption spectrum which occurred after exposure of the leaves to various periods of illumination. He found that the red absorption maximum of newly formed chlorophyll is at 684 mμ. After 10-20 minutes the peak

shifted to 673 mμ, and after 1 hour it shifted again to a final position at 677 mμ, which is the characteristic absorption maximum of chlorophyll a in mature green leaves. These spectral shifts were interpreted as being caused by association of newly formed chlorophyll with its protein carrier, and the integration of the pigment-protein complex with the chloroplast structure.

The complex of pigment plus protein carrier has been given the name 'holochrome' (Smith and Young, 1956). Analyses of chlorophyll holochrome preparations have shown that the carrier part of the complex is about 70% protein and 30% lipid (Kupke and French, 1960), suggesting that the carrier is a lipoprotein.

2. The Chloroplast.

A) Structure.

The chloroplast is the cytoplasmic organelle which contains the chlorophyll and carotenoid pigments. In lower plants chloroplasts are found in a variety of shapes and sizes. In the higher plants, however, they are usually ellipsoidal or lens-shaped, and average 5μ in diameter and 2-3μ in thickness. The number of chloroplasts per cell varies widely with the species, ranging from one in some of the algae to more than a hundred in certain higher plants, but they generally account for 20-30% of the dry weight of leaves. (Thomas, 1960).

The mature green chloroplast is surrounded by a semi-permeable double membrane. Internally it is composed of layers of double membrane structures or lamellae which extend parallel to the long axis of the chloroplast and are embedded in a granular proteinaceous stroma. In certain regions the lamellae may appear to be more dense, the dense regions of several lamellae coinciding with one another to form darker areas called grana. These dense regions of the lamellae are disk-shaped, so that a granum is a stack or cylinder of disks.

Various interpretations of the fine structure of lamellae and grana, based on electron micrographs, are to be found in the literature (Granick, 1961; von Wettstein, 1958; Weier and Thomson, 1962). Differences in interpretation may be due to variation between species, to different methods of fixation and embedding of material for electron microscopy, or to physiological variation arising from changing environmental conditions (Weier and Thomson, 1962). However, the view that the grana regions are merely thickenings of the lamellae (von Wettstein, 1958) has been criticised by Weier and Thomson (1962), who describe them as flattened hollow disks. They consider that the contents of the disks are not in contact with the granular stroma and that the disks contain no electron dense material. The latter view is supported by a survey of electron micrographs in the literature representing contributions from numerous laboratories using a wide variety of techniques.

Weier and Thomson (1962) also point out that the appearance of grana in living and fixed chloroplasts under the light microscope does not agree with the grana structures seen in electron micrographs. Under the light microscope grana in side view are small and spindle-shaped (Weier, 1961) whereas under the electron microscope they appear rectangular and sometimes extend from the top to the bottom of the chloroplast. The cause of this disparity is not clear. In surface view the grana are always circular whether looked at with the light or the electron microscope.

Both the lamellae and the grana are lipoprotein. Some evidence for this has been provided by studies using the polarising microscope (Granick, 1961). The birefringence and refractive index of chloroplasts reveal the presence of protein layers. When tissues are fixed with osmium to prevent loss of lipids and then viewed again with the polarising microscope, the lipid molecules appear to be orientated perpendicularly to the protein layers. Further evidence comes from electron microscope studies of osmium-fixed leaf material. The lamellae have electron dense layers which are attributed to lipid, and less dense layers thought to be protein. The fact that chloroplasts are very rich in lipid (23-35% of their dry weight, Granick, 1961) agrees well with the concept of a lipoprotein lamellar structure.

It can be seen under the light microscope that chlorophyll

is concentrated in the grana regions of the chloroplast, and it has been demonstrated (Olson and Engel, 1958) by means of absorption microscopy to be distributed along the intergrana lamellae also. This is still further evidence of a pigment-lipoprotein complex in the chloroplast. It seems probable that the chlorophyll molecules are spread in a single layer over the lamellae with the phytol groups embedded in the lipid layer. Whether the porphyrin nuclei are embedded in the protein layer or lie in contact with the stroma is not clear, owing to incomplete knowledge of the architecture of the lamella (Sager, 1958). In an attempt to relate chloroplast structure to photosynthetic function, Calvin (1958) proposed a hypothetical model for the arrangement of pigments, lipid and protein in the lamella.

In addition to the structures already mentioned, chloroplasts characteristically contain dense spherical bodies, 50-5000Å in diameter, embedded in the stroma between the lamellae (von Wettstein, 1957; Buvat, 1958). These are called osmiophilic granules, or globuli. Their function and chemical nature are not certain but they probably contain lipid (Park and Pon, 1961) and possibly carotenoid pigments as well (von Wettstein, 1958). They have been observed to accumulate in large numbers at a certain stage of chloroplast development in a chlorophyll mutant of barley, and also in degenerating chloroplasts (von Wettstein, 1958). In normal barley plants the globuli appear at an early stage in plastid

development, and von Wettstein (1957b) has suggested they may play a part in the formation of lamellae and grana.

B) Development.

The morphological development of chloroplasts has been studied in several species of plants by various investigators (eg. Hodge, et al., 1956; von Wettstein, 1958; Buvat, 1958; Gerola, et al., 1960; Epstein and Schiff, 1961). Chloroplasts arise in meristematic cells from proplastids. These small bodies are enclosed by a double membrane and have a dense proteinaceous stroma, but are undifferentiated internally. They are distinguished from young mitochondria in being slightly larger and often containing globuli (Buvat, 1958).

Development of proplastids into mature chloroplasts involves both an increase in size and an internal differentiation into lamellae and grana. The process of differentiation appears to be similar in all tissues studied, beginning with budding from the inner layer of the proplastid membrane of spherical or elongate vesicles and the fusion of these vesicles in layers to form parallel double lamellae. According to von Wettstein (1957b) grana may form in dicotyledons before the lamellar system is well developed, whereas in monocotyledons they do not appear until the lamellae have formed continuous layers. The development of lamellae and grana accompanies the accumulation of chlorophyll and during this period the plastids enlarge considerably. The number of lamellae and the size of grana

increase and finally the plastid assumes the ellipsoidal or lens shape of a mature chloroplast.

Chloroplast development has also been studied in etiolated tissues following exposure to light for various periods of time. The process appears to be somewhat different from that described above for development under natural conditions. In the dark, vesicles begin to form in the proplastids just as in the light, but instead of becoming organised into lamellae, they accumulate and form what is called a prolamellar body. The etiolated plastids increase in size but do not reach mature proportions. The prolamellar body may assume a "crystal lattice" structure in which the lattice is made up of a network of tubules formed from the vesicles. More than one prolamellar body may be formed per plastid. When the etiolated plant is exposed to light chlorophyll formation begins and lamellae arise out of the vesicular mass within a few hours. The grana appear later and eventually the plastids reach full size with a structure indistinguishable from the normal. According to Klein and Poljakoff-Mayber (1961), when plastids isolated from etiolated bean leaves are exposed to as little as 0.5 minutes of light and immediately fixed, changes can be observed in the prolamellar body. The fact that lamellae and grana had not been observed to form until chlorophyll was present in the plastid in substantial amounts led to the suggestion that their development involves the chlorophyll molecule directly.

For example, Butler (1961) proposes that chlorophyll molecules may act as a glue, cementing the lipid and protein layers together. This view is contradicted by von Wettstein's earlier finding (1958) that etiolated barley leaves kept in darkness for very long periods of time may begin to form lamellae in the absence of chlorophyll. Similarly, a few lamellae are able to form in a certain chlorophyll mutant of barley which contains neither chlorophyll nor protochlorophyll (von Wettstein, 1959). No grana form in either case, however, and lamellar formation is limited. Thus, while neither protochlorophyll nor chlorophyll is necessary for lamellar formation, chlorophyll is essential for grana development.

C) Biochemistry.

The composition of the mature green chloroplast on a dry weight basis is as follows: protein, 40-50%; lipid, 23-35%; chlorophylls _a_ and _b_, 5-10%; carotenoids, 1-2% (Granick, 1961). It also contains both ribonucleic acid (RNA) and desoxyribonucleic acid (DNA) (Chiba and Sugahara, 1957; Cooper and Loring, 1957).

Etiolated plastids are relatively poor in protein (de Deken-Genson, 1954; Mego and Jagendorf, 1961). As described in the previous section, exposure to light of etiolated plastids results in an increase in size and in the internal differentiation of lamellae and grana. There is a concomitant large increase in protein during this process (de Deken-Grenson, 1954; Brawerman and Chargaff, 1959;

Mego and Jagendorf, 1961), presumably due to the requirement for lipoprotein to build lamellae and grana, and probably for enzymes as well. This increase in plastid protein is accompanied by a corresponding decrease in the total free amino acid nitrogen of the cell (de Deken-Grenson, 1954).

The question has been raised as to whether the synthesis of plastid protein during greening takes place entirely within the plastid itself, or whether some or all of the protein is formed in the cytoplasm and transferred to the developing plastid (Brawerman and Chargoff, 1959; Granick, 1961). Stephenson, et al. (1956) established that isolated chloroplasts are able to synthesise protein from supplied amino acids, and Bove and Raacke (1959) demonstrated the presence of amino acid-activating enzymes in chloroplasts. Also, Lyttleton (1962) recently isolated ribosomes from spinach chloroplasts which appear to be physically and chemically distinct from cytoplasmic ribosomes. There can be little doubt, therefore, that at least some protein synthesis occurs within the plastid. However, Brawerman and Chargaff (1959) show that in Euglena some transfer of cytoplasmic protein into the plastid also occurs during chloroplast formation.

To date, studies of amino acid incorporation by greening chloroplasts have either involved the use of artificial mixtures of amino acids supplied to isolated chloroplasts, or have been limited to measurements of the total free amino acid content of

whole tissues. No reports have appeared of qualitative or quantitative measurements of individual free amino acids during incorporation. Folkes (1951) studied the amino acid metabolism of germinating barley. He found that the two main endosperm proteins of Barley, hordein and hordenin are richer than tissue proteins in amides, glutamic acid and proline, and are deficient in the basic amino acids. Over 60% of hordein is in glutamine and proline residues. During germination there is synthesis of aspartic acid, alanine, lysine, arginine, and to a lesser extent glycine, isoleucine, tryptophane and threonine (as well as chlorophyll and purines).

In these syntheses 90% of the nitrogen involved is derived from amides, glutamic acid and proline. Decreases in phenyl alanine and serine are also observed. Since proline can be converted to glutamic acid by oxidation, glutamic acid and glutamine appear to have a central position in protein synthesis.

Thomas (1960) has reviewed the work that has been done on amino acid oomposition of leaf proteins. There appears to be little difference in composition from one family of plants to another, or between cytoplasmic and chloroplastic proteins. Lysine is an exception, however, being significantly higher in the cytoplasmic proteins of plants (Yemm and Folkes, 1953). Yemm and Folkes also showed that in barley there is little change in leaf protein composition from the young seedling stage to maturity.

Studies by Mego and Jagendorf (1961) show that the increase in protein content and size of etiolated bean plastids during greening is accompanied by an increase in non-pigment lipid. This is presumably required for the lipoprotein structures of the chloroplast. Brawerman and Chargaff (1959; 1961) in studies using colourless Euglena cells, demonstrated that greening was accompanied by an increase in plastid and microsomal RNA, which precedes the increase in plastid protein. Moreover, the nucleotide pattern of RNA in both microsomes and plastids was altered, suggesting that formation of new RNA of a specific character took place. This is in agreement with Lyttleton's (1962) finding that ribosomes of the chloroplast have different base ratios than those of the cytoplasm.

3. Chlorophyll Mutants.

The study of chlorophyll mutants has been helpful in elucidating the processes of chlorophyll biosynthesis and accumulation. Chlorophyll mutations occur naturally in a wide variety of plants, and can readily be induced by ionizing radiations or by chemical agents.

Chlorophyll mutants are classed into three main groups according to colour: albina (white or pale yellow), xantha (yellow or yellow-green), viridis (pale green). Albino mutants contain little or no yellow pigment, and though they form chlorophyll in small amounts they are unable to retain it. Xantha mutants contain

carotenoids but are low or lacking in chlorophyll. Viridis mutants contain both chlorophyll and carotenoids in amounts which are below normal. The chlorophyll mutants used most extensively in studies on chlorophyll formation have been those of corn and barley mainly of the albina and xantha types. Chlorophyll content is known to be controlled in these plants by a large number of genes, and the variety of mutants found within each of the three main groups is very great.

In a study of pigment formation in corn mutants, Koski examined the protochlorophyll level in dark-grown plants of six different types of mutant and compared them with their normal sibs (Smith and Young, 1956). She found that all mutants except one were low in protochlorophyll, but that one mutant had more than the normal amount. None lacked protochlorophyll altogether, so the biosynthetic pathway as far as protochlorophyll must have been intact in all cases. Illumination for five minutes of the mutant seedlings always converted the same proportion of protochlorophyll to chlorophyll a. Continued illumination had various effects on the chlorophyll content of the different mutants. In most the chlorophyll content increased but not to the same extent as in the normals. In one mutant it decreased and in another chlorophyll was lost completely. The conclusion drawn was that chlorophyll deficiency results in two ways: by insufficient protochlorophyll production and by increased chlorophyll destruction.

A study of albino mutants of corn was carried out by Smith, et al. (1957; 1959) in an attempt to establish the nature of the factor(s) responsible for chlorophyll stability or instability in the leaf. They examined the ability of mutants to esterify newly formed chlorophyllide with phytol and found that while most albinos had low phytolising ability, one was normal. The shift in the red absorption maximum of newly formed chlorophyll a *in vivo*, thought to be due to stabilisation by protein, (Shibata, 1957), was also examined. Most of the albinos showed little if any shift, but one was normal in this respect. An examination of carotenoid pigments as possible protectors of chlorophyll from photodestruction showed that although most mutants had a very low carotenoid content some were still relatively rich in it. Yet all mutants bleached on continued illumination. The authors concluded that none of these factors alone accounts for the inability of mutants to accumulate chlorophyll.

Von Wettstein (1957; 1958) examined the fine structure of plastids from different types of chlorophyll mutants. He found that there are blocks at various stages in the structural development of chloroplasts which are related to the degree of pigment deficiency. Albino mutants are blocked in the early stages of plastid development. The plastids are able to form vesicles, and the vesicles may aggregate into a prolamellar body or even line up in rows, but they soon break down again and development proceeds

no further. The xantha mutants are blocked at later stages of chloroplast development. Xantha-3 of barley, for example, produces vesicles, a prolamellar body and a few primary layers, but further development is arrested. Large numbers of globuli form and later disappear accompanied by pigment destruction. Xantha-10 of barley, which has neither protochlorophyll nor chlorophyll (von Wettstein, 1959) can form lamellae similar to those found in normal plastids under conditions of prolonged etiolation, but no grana are produced. Some viridis mutants develop a limited lamellar system with a few small grana. Others produce completely normal plastids.

METHODS AND MATERIALS

1. Plant Material

The chlorophyll mutant used in these studies was produced from Gateway barley by chemical treatment. In breeding studies (Walker, et al., 1962) it behaved as a simple recessive and is described as viable yellow ($^{y}vi_2$), with the mutant gene on chromosome I. The studies presented here show that it belongs, in fact, to the viridis group of mutants and may be described as virescens, i.e. pale mutants which recover greenness with age.

Normal Gateway barley seed was obtained from field-grown plants, and mutant seed was obtained from plants grown in the greenhouse. This initial lot of mutant seed was approximately 20% higher in nitrogen content per unit weight than the normal. Plant material grown from these batches of seed was used for some of the pigment studies and all of the microscopic investigations. For the remainder of the pigment studies and for the protein, amino acid and nucleotide analyses the plant material was grown from normal and mutant seed produced under identical conditions in a growth chamber. The nitrogen contents of normal and mutant seed under these conditions were approximately the same (normal = 29 mg. N/g; mutant = 31 mg. N/g). The plants grown from this seed did not appear to differ in any way from plants grown from the original stock.

All seed was surface sterilized before sowing by soaking for 5 minutes in a 1% suspension of a commercial fungicide (Orthocide). For studies on light-grown material seed was sown in pots or in wooden flats in a soil : peat : sand mixture in the proportions 3 : 1 : 1. The flats were placed in a growth chamber at 70°F, 50% humidity and 1500 f.c. light produced by daylight fluorescent bulbs. The photoperiod was 18 hours. For one fractionation experiment plant material was grown in the greenhouse with supplemented lighting to give an 18 hour photoperiod. Although these normal and mutant plants were grown under identical conditions, temperature, humidity and light intensity could not be maintained at a constant level. When etiolated plants were required, seed was sown in Perlite in large pyrex baking dishes. The Perlite was moistened with Hoagland's No. 1 solution supplemented with some micronutrients, and the dishes were placed in a ventilated dark chamber at 20$\pm$ 2°C. On the third or fourth day they were watered with distilled water. This operation was carried out with the aid of a green safelight with a Farrand interference filter #110323 absorption 498 mμ, half band width 12 mμ. Seedlings were used for experimentation at the end of 7 days when the normal and mutant were between 12 and 15 cm. in length, and had been emerged for 4 days.

The 2 - to 3- day seedlings used in electron microscope studies were grown on moist filter paper in covered glass beakers

under artificial light. Those used in light microscope studies were grown in soil.

2. Pigment Analysis.

A) Pigment Determinations.

For estimation of pigment concentration in light-grown material duplicate samples of 5 leaves each were harvested, weighed and chopped into small glass mortars containing about 10 ml. pure acetone and small amounts of washed sand and $CaCO_3$. The tissue was ground briefly and the acetone extract decanted. The residue was extracted again three times until a total of 35 ml. acetone had been used. The four extracts were pooled and centrifuged in a ~~small~~ Servall angle centrifuge at 10,000 g. for 10 minutes. The final cleared extract was decanted, the volume measured, and the absorption spectrum between 400 and 700 $m\mu$ recorded in a Beckman DK1 recording spectrophotometer, using 1 cm. cells.

Pigment determinations in etiolated material were made on duplicate samples of 20 leaves each. Seedlings were harvested in the dark using the green safe light described previously. They were cut 5 cm. from the leaf apex and the excised portions were placed in light-tight weighed tins and their weight recorded. Pigments were extracted with 35 ml. pure acetone as described above. All operations up to centrifugation were carried out under

dim green light. The final volume of the extract was measured and its spectrum recorded in a 10 cm. Beckman cell.

The use of pure acetone as solvent in measuring pigment concentrations avoided the need to transfer the pigments to ether and made possible more rapid determinations and better recoveries. Pure acetone rather than 80% was used because protochlorophyll estimations were necessary in etiolated material and the absorption coefficients given for this pigment in the literature (Koski and Smith, 1948) are for pure acetone. The formulae for calculation of pigment concentration in extracts of light and dark-grown plants were derived using MacKinney's (1940) specific absorption coefficients for chlorophylls a and b in pure acetone, and those of Koski and Smith (1948) for protochlorophyll. These absorption coefficients, the derivations and the formulae are given in the Appendix.

Carotenoid concentrations were determined in the acetone extracts of both light and dark-grown plants using von Wettstein's (1957a) formula (see Appendix).

B) Chromatography of Pigments.

Pigments from light-grown leaves were extracted with acetone and transferred to ether in a separatory funnel by the method of Koski, French and Smith (1951). The ether extract was concentrated in a stream of air and a small amount applied to one corner of a piece of Whatman No. 1 filter paper 22 x 22 cm. which had

previously been washed with Skellysolve B and air dried. The paper was rolled and fastened into a cylinder. Two dimensional chromatography was carried out by the ascending method of Lind, et al., (1953) as follows: 1st dimension: 1) acetone - to bring pigments into a line at the top of the original spot, 2) Skellysolve B - until solvent front was 20 cm. from the bottom, 3) 10% n-propanol in Skellysolve B - until solvent front was 20 cm. from the bottom. 2nd dimension: 4) 25% chloroform in Skellysolve B - until solvent front was 16 cm. from the bottom.

3. Microscopic Studies.

A) Light Microscopy.

Normal and mutant seedlings were taken 2 or 3 days and 11 or 12 days after emergence. The 2- to 3-day seedlings were $1\frac{1}{2}$ - 3 cm. in height. They were excised from the seeds, placed in 0.75M sucrose on a microscope slide, and a cut made 3 mm. from the apex. Tissue was teased away from the cut area in order to isolate thin regions. At 11 and 12 days after emergence the primary leaves were 8-10 cm. in length. They were treated in the same manner except that pieces of tissue were taken from an area approximately half-way down the leaf. Tissue fragments were examined and photographed with a Zeiss photomicroscope.

Fixation and embedding of leaves for light microscopy was carried out using 3 mm. pieces cut from the central portions of

leaves at 4 and 11 days after emergence. The leaf pieces were fixed in Rawlin's solution and dehydrated in a series of ethanol-n-butanol-water mixtures of increasing alcohol concentration, ending with 3 changes of absolute n-butanol. The pieces of leaf were transferred to a 1:1 mixture of absolute n-butanol : heavy paraffin oil and left for several hours. After 2 changes in parawax they were embedded in tissue mat. Sections 12μ thick were cut, hydrated and stained with Delafield's haematoxylin solution and aqueous safranin.

B) Electron Microscopy.

For electron microscope studies small pieces of tissue (1 mm^2) were cut from the same regions of 2- to 3-day seedlings and 12-day leaves as described above. The cutting was carried out in a fixing medium of 1% osmium tetroxide in acetate-veronal buffer, pH 7.2, containing 0.01 M $CaCl_2$ and 0.2 M NaCl. Tissues were fixed for 4 hours at room temperature, transferred to 0.5% uranyl acetate in the same buffer for 1 hour and washed briefly with buffer. Dehydration in an acetone series and embedding in vestopal W was carried out according to the method of Kellenberger, et al. (1958) except that propylene oxide was used as a wash after dehydration, and was substituted for acetone in the vestopal W mixtures. Thin sections were cut using a Porter-Blum microtome and handled as described by Fitz-James (1960). They were examined

with a Philips 100A electron microscope with an objective aperture of 40μ and an accelerating voltage of 60 kv. Photographs were taken at an initial magnification of 4,000-17,000x.

4. <u>Biochemical Studies</u>.

A) Fractionation.

Primary leaves were harvested, weighed and finely homogenised in ice-cold 0.33M KCl - 0.02M phosphate buffer, pH 7.0. The homogenate was squeezed through 4 layers of cheesecloth and the volume measured. (A very small amount of octyl alcohol was used to remove foam.) The homogenate was agitated and four aliquots of 5 ml. each were transferred to centrifuge tubes. Centrifugation was carried out at 0°C. for 15 minutes at 37,000 g to collect particulate matter. The supernatant was precipitated with 0.5 ml. 20% trichloroacetic acid (to bring the pH below 2) at room temperature and the precipitate collected by centrifugation at 6,000 g for 10 minutes. The final supernatant was made to 10 ml. with distilled water. Thus three fractions were obtained: 1) particulate fraction, 2) acid-insoluble fraction, 3) acid-soluble fraction.

B) Nitrogen Determination.

The total nitrogen of the various fractions was determined by the microkjeldahl method. Aliquots of fraction 3 were taken and fractions 1 and 2 were transferred quantitatively to kjeldahl

flasks. Samples were digested with 1 ml. $HgSO_4$, 3 ml. conc. H_2SO_4 and 1 g. K_2SO_4. After digestion and cooling, 15 ml. NaOH-$Na_2S_2O_3$ (50% : 5%) were added to the digests in a distillation apparatus, and the distillate was collected in 5 ml. 4% HBO_3 solution containing 2 drops of 0.1% bromcresol green in ethanol. Titration was carried out against freshly standardized HCL. Determinations for each fraction were made in quadruplicate and results are expressed as the averages and the standard error of the means.

C) Free Amino Acid Analysis.

An aliquot of the acid-soluble fraction was evaporated to dryness in a flash evaporator at 45-55°C, taken up in distilled water and evaporated again to dryness. A few ml. distilled water were added to dissolve the residue, the pH was adjusted to between 7 and 8 with NaOH, and the sample was left standing at room temperature for 4 hours. This step converts any cysteine present to cystine. The solution was adjusted to pH_2 with HCL and the sample brought to a suitable volume with 0.2N Na-citrate buffer, $pH_{2.2}$. Samples were stored frozen until used. Amino Acid analysis was carried out with a Beckman/Spinco model 120 Amino acid Analyser.

D) Nucleotide Analysis.

The ribonucleotide content of fraction 3 was determined by the method of Mejbaum (Davidson and Chargaff, 1955) for ribose as follows: 1 vol. unknown plus 1 vol. orcinol reagent (0.1% orcinol - 0.1% $FeCl_3.6H_2O$ in conc. HCL, sp. gravity 1.19) was

heated for 20 minutes in a boiling water bath and the optical density read at 670 mu. Readings were compared with a standard curve obtained using yeast RNA (see Appendix). Nitrogen contents were calculated assuming that nitrogen accounts for 15% by weight. In these estimations no attempt was made to correct for possible interference by non-nucleotide sugar. It should be noted that the method assumes that RNA and the free nucleotides were comparable in base composition.

RESULTS

1. Pigment Studies.

A) Concentrations.

The mutant was found to be sensitive to conditions of high light intensity and high temperature, to the extent that it bleached and wilted beyond recovery when planted in the field or greenhouse during the summer months. Low light intensities (below 1000 f.c.) and low temperatures (below 60°F.) resulted in decreased pigment content in both normal and mutant. The conditions of the growth chamber (1500 f.c., 70°F.) resulted in relatively vigorous mutant plants which developed the highest pigment levels observed and could readily be grown to maturity.

At best, the growth and maturation of the mutant in the light were slower than that of the normal. During the first two weeks of growth the mutant seedlings were slightly shorter than the normal (Fig. 1). At 2 months (Fig. 2) the gross morphology was clearly different. The mutant had longer, broader leaves and shorter internodes than the normal and showed no sign of heading. It was at least a month later in setting seed.

In the growth chamber, the mutant seedling at emergence was a pale yellow-green, distinct from the bright green normal sprouts. With age, however, the colour of the mutant deepened until, at the 2-week stage, it was almost as green as the normal. This process of gradual deepening of colour occurred in each new


NORMAL
MUTANT

Figure 1. Normal and mutant Gateway barley seedlings from the growth chamber at 11 days after emergence.

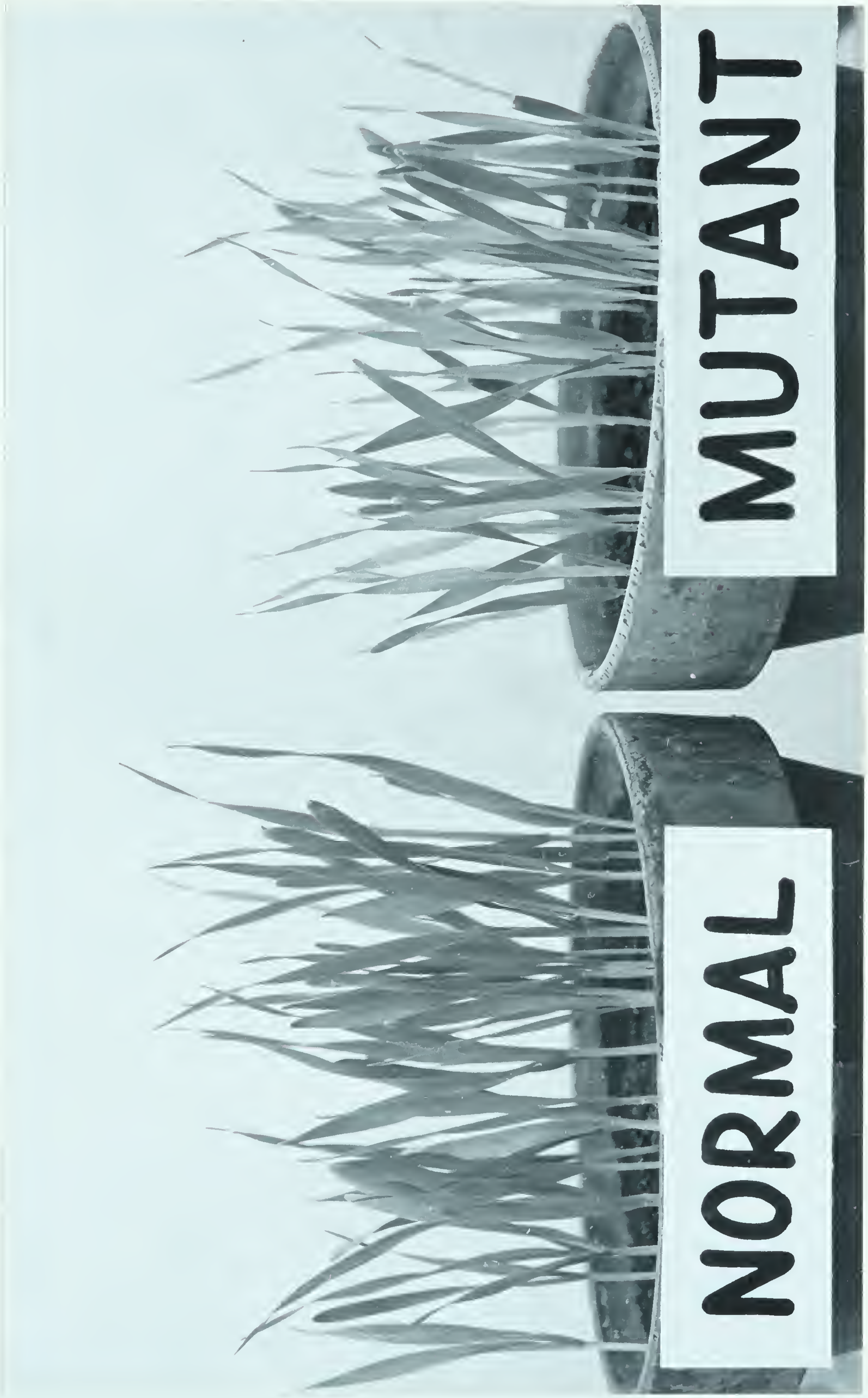
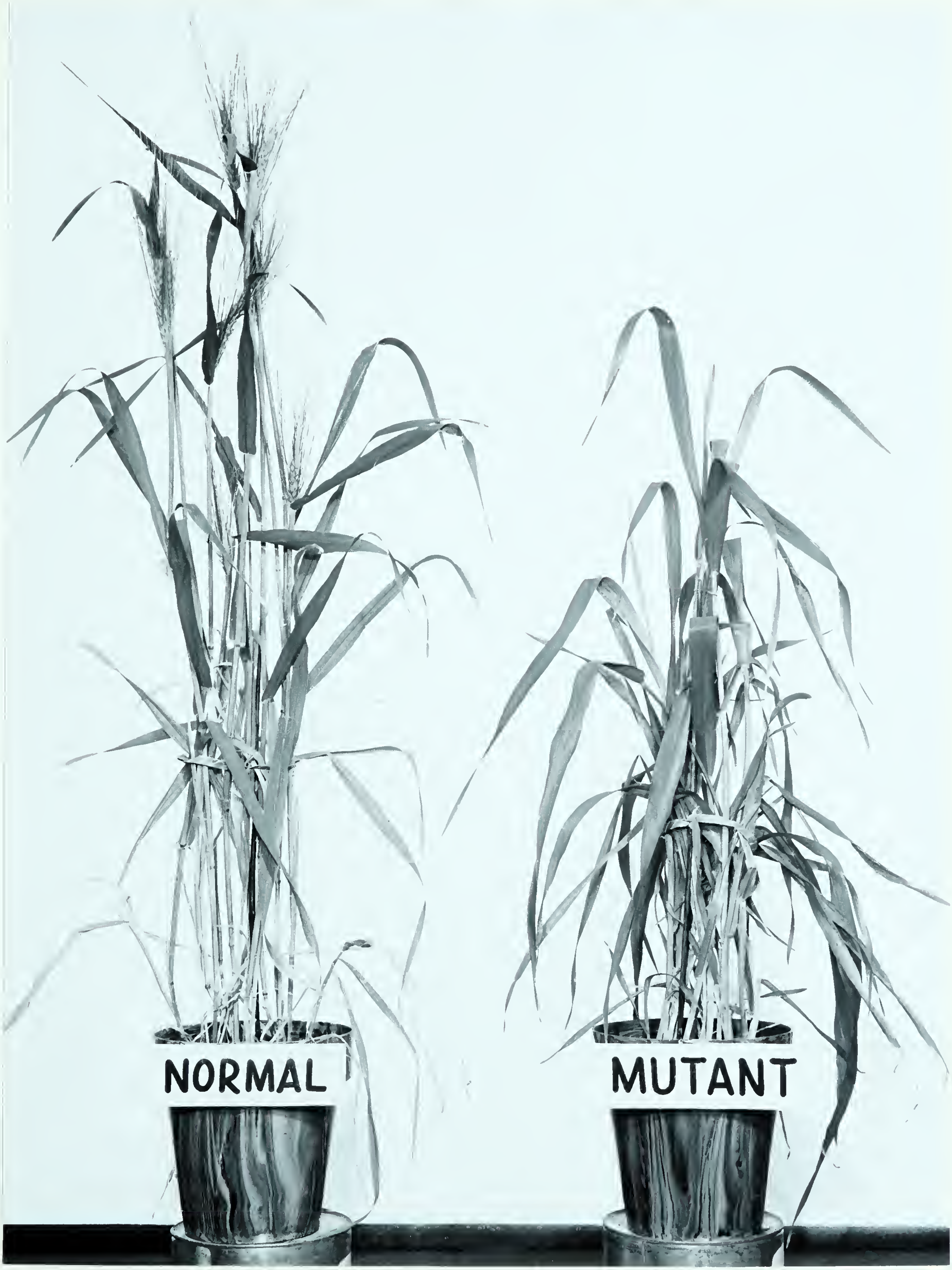

MUTANT
NORMAL

Figure 2. Normal and mutant Gateway barley plants from the growth chamber at 2 months.


NORMAL
MUTANT

leaf as it developed, beginning at the leaf tip. Thus when the older leaves of a plant were fully green, the new leaves were still yellow-green (Fig. 1). In studying the pattern of pigment recovery, therefore, only the primary leaves were used.

The concentration of chlorophyll per gram fresh weight of leaf as a function of the age of tissue is given in Figure 3. Both normal and mutant seedlings showed a considerable increase in chlorophyll in the first two weeks of growth. The mutant, however, began with much less chlorophyll a and almost no chlorophyll b, and it never accumulated chlorophyll to levels found in the normal. Maximum recovery by 15 days was 75-80%, and the mutant was 2 to 3 days later than the normal in reaching its maximum pigment level. The mutant was lower in carotenoid pigment also, containing approximately 50% of the normal amount at the 3-day stage and 75% at two weeks (Table I). An examination of the absorption spectra of acetone extracts of normal and mutant leaves showed that at every stage the mutant had the same complement of chloroplast pigments as the normal. This was supported by chromatographic studies of pigment extracts, which showed that no unusual pigments were present and no pigments were lacking.

When grown in darkness normal and mutant seedlings developed at about the same rate. Growth ceased in both at the end of 6 days in the absence of added carbohydrate. Pigment concentrations in these etiolated plants were measured in several experiments and some of the

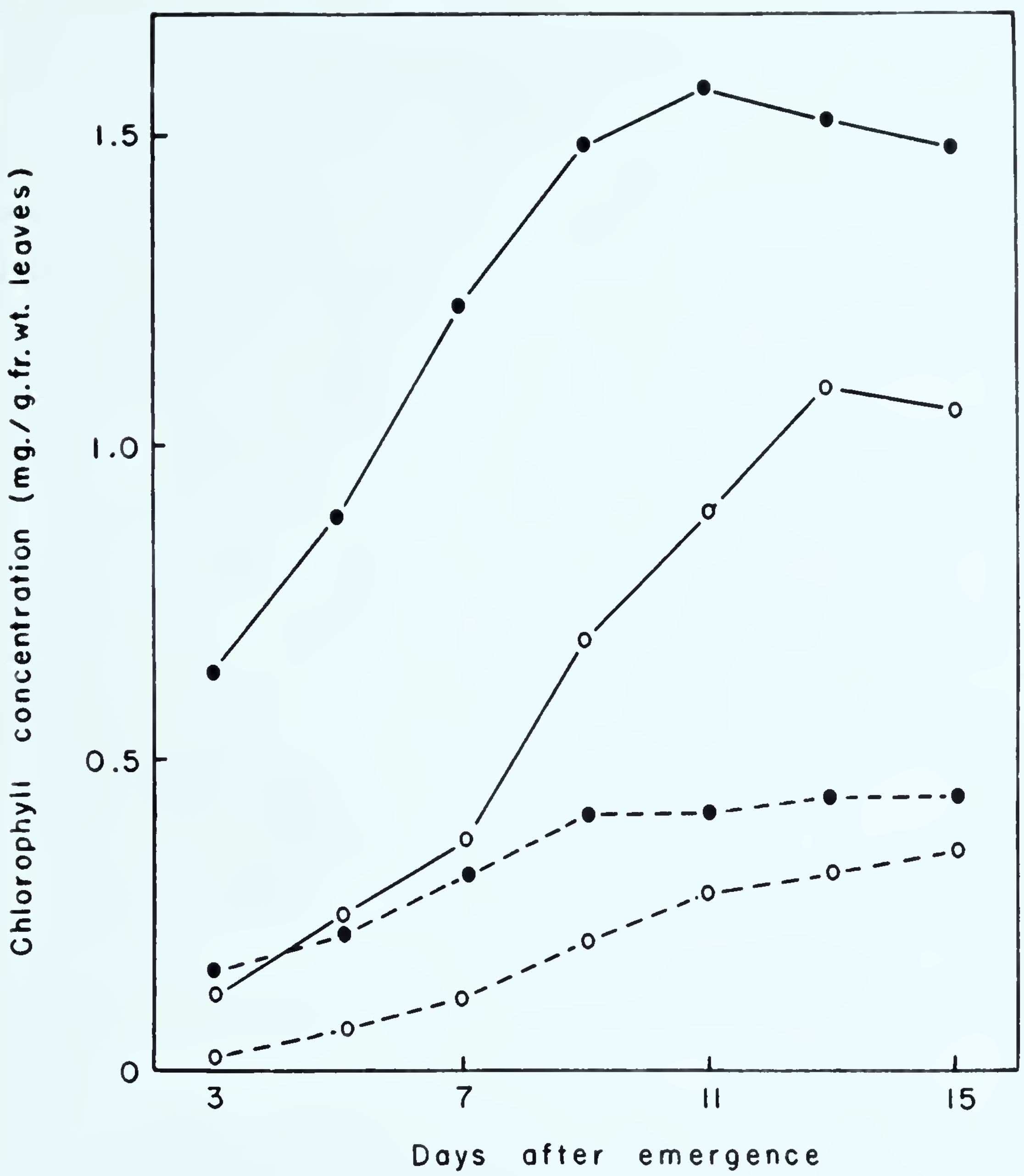


Figure 3. Concentration of chlorophyll a and b in primary leaves of normal and mutant barley, measured at 2-day intervals from 3 to 15 days after emergence. Solid circles - normal; open circles - mutant; solid lines - chlorophyll a; broken lines - chlorophyll b.

TABLE I

CAROTENOID CONTENT OF PRIMARY LEAVES OF NORMAL AND MUTANT LIGHT-GROWN BARLEY PLANTS, MEASURED AT 2-DAY INTERVALS UP TO 15 DAYS AFTER EMERGENCE

	Days after emergence						
	3	5	7	9	11	13	15
	Carotenoids (ug./g.fr.wt.)						
Normal	180	174	219	252	226	201	204
Mutant	88	107	110	133	145	149	146
	% (M/N x 100)						
	49	61	51	57	64	74	72

TABLE II

PROTOCHLOROPHYLL AND CAROTENOID CONTENT OF
PRIMARY LEAVES OF NORMAL AND MUTANT DARK-GROWN BARLEY PLANTS
AT 4 DAYS AFTER EMERGENCE

	Concentration (ug./g.fr.wt.)						
	Protochlorophyll			Carotenoids			
	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 4	Exp. 5
Normal	12.4	11.1	13.6	115	105	118	120
Mutant	9.3	8.8	8.9	89	62	80	70
	% (M/N x 100)						
	75	79	65	74	60	68	58

data are collected in Table II. Protochlorophyll concentrations were lower in the mutant, varying between 65 and 80% of the normal concentrations. The carotenoid content was less than that of the light-grown leaves (cf. Table I), and the mutant always contained less than the normal. Thus all chloroplast pigments were reduced in quantity in the mutant at all the stages of development studied in light-grown and in etiolated plants.

B) Syntheses.

The accumulation of chlorophyll was measured in 4-day normal and mutant excised etiolated leaves after exposure to continuous light. The results are given in Figure 4. The pattern was the same in both normal and mutant as that described by Koski (1950) for corn seedlings. After the initial rapid conversion of protochlorophyll to chlorophyll <u>a</u> there was a period of an hour during which no more chlorophyll was formed. Then the second, accelerated phase of synthesis began in both normal and mutant and at the same time chlorophyll <u>b</u> was first detectable. At no time up to 4 hours was the rate of accumulation as rapid in the mutant as in the normal.

The rate of protochlorophyll formation was also measured in 4-day normal and mutant etiolated seedlings from which the original protochlorophyll had been removed by photo-conversion to chlorophyll <u>a</u>. The conversion was effected by exposure to 160 f.c. light for 10 minutes. In a number of preliminary experiments with detached leaves on moist filter paper this light treatment resulted

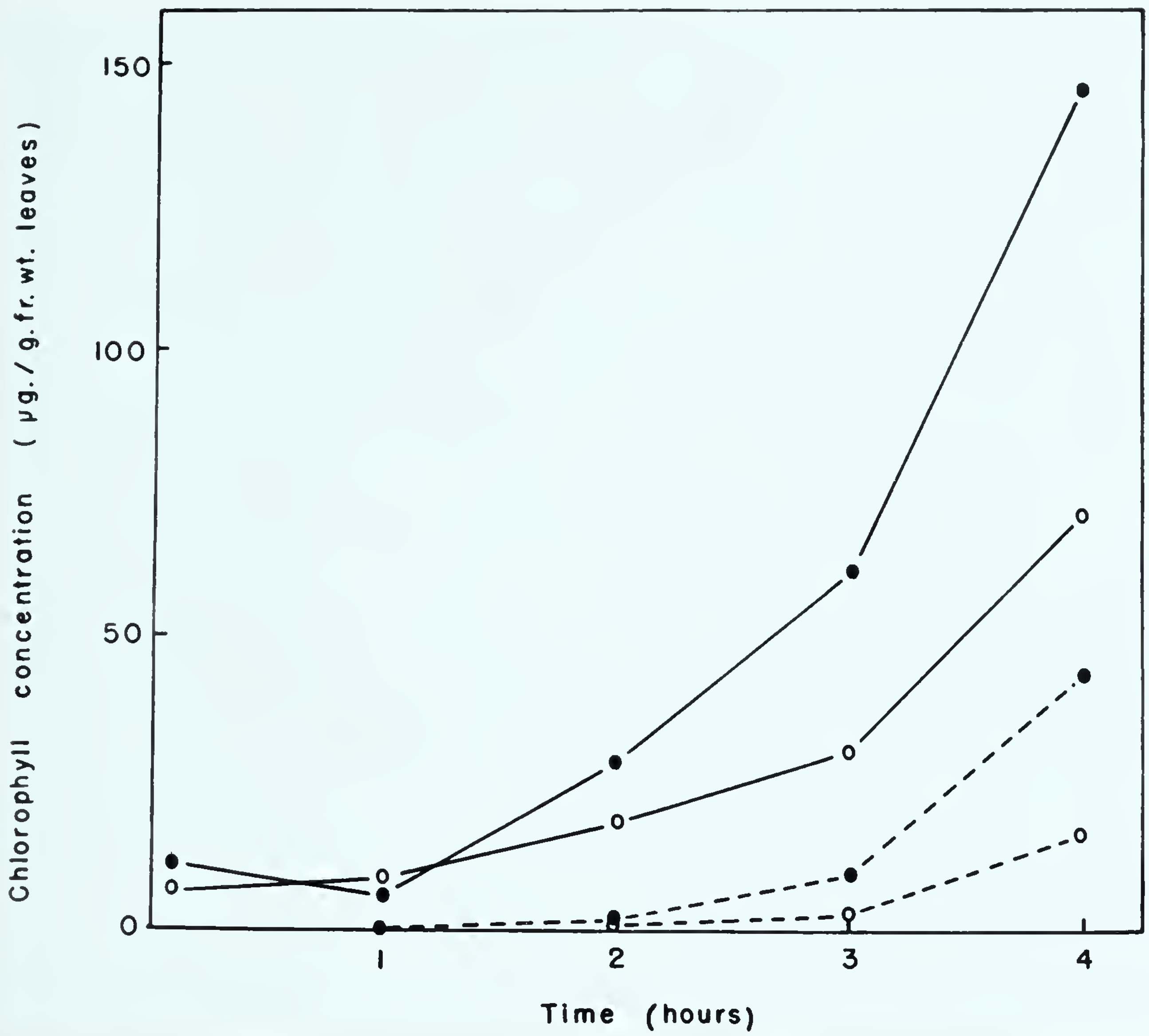


Figure 4. Levels of chlorophyll a and b in 4-day etiolated leaves of normal and mutant barley at periods of up to 4 hours continuous light (160 f.c.). Solid circles - normal; open circles - mutant; solid lines - chlorophyll a; broken lines - chlorophyll b.

in 95+% transformation in both normal and mutant. A few experiments were carried out using intact seedlings and in these there was usually no more than 60 to 70% transformation (even with illuminations of 500 f.c.), possibly because all leaves were not uniformly illuminated. Seedlings were then incubated in darkness and protochlorophyll levels were measured at intervals. Results of two typical experiments (one on detached leaves and one on intact seedlings) are given in Table III. Although initial protochlorophyll levels after illumination were different, under the two sets of conditions the rate of formation of protochlorophyll was slower in the mutant and it never accumulated to levels found in the normal (cf. Table II).

2. Chloroplast Development.

Photomicrographs of fixed normal and mutant leaf material are shown in Figures 5 and 6. At the 4-day stage the mutant chloroplasts (Fig. 5B) appear to be smaller than the normal (Fig. 5A) and are not as clearly delineated. Many have been broken during the fixation process. When seen in side view (i.e. those closely appressed to cell walls) they are flatter than normal chloroplasts. By the 11th day the mutant chloroplasts have almost recovered (Fig. 6B). They are larger, more distinct and more darkly stained. Few appear to have been damaged in fixation. They are closer to the normal (Fig. 6A)

TABLE III

PROTOCHLOROPHYLL SYNTHESIS IN 4-DAY ETIOLATED NORMAL AND MUTANT BARLEY SEEDLINGS IN WHICH ORIGINAL PROTOCHLOROPHYLL LEVELS HAD BEEN DEPLETED BY EXPOSURE TO LIGHT FOR 10 MINUTES

Time after Illumination (hours)	Protochlorophyll Conc. (ug./g.fr.wt.)	
Exp.1 (detached leaves)	Normal	Mutant
0	0.6	0
3	8.1	2.9
6	9.0	4.0
16	8.7	3.9
Exp.2 (intact seedlings)		
0	7.2	5.6
7	10.9	7.2

Figure 5. Photomicrographs of transverse sections cut through the mid-vein region of 4-day primary leaves of Gateway barley fixed in Rawlin's solution. A - normal; B - mutant. Mag. x 270.

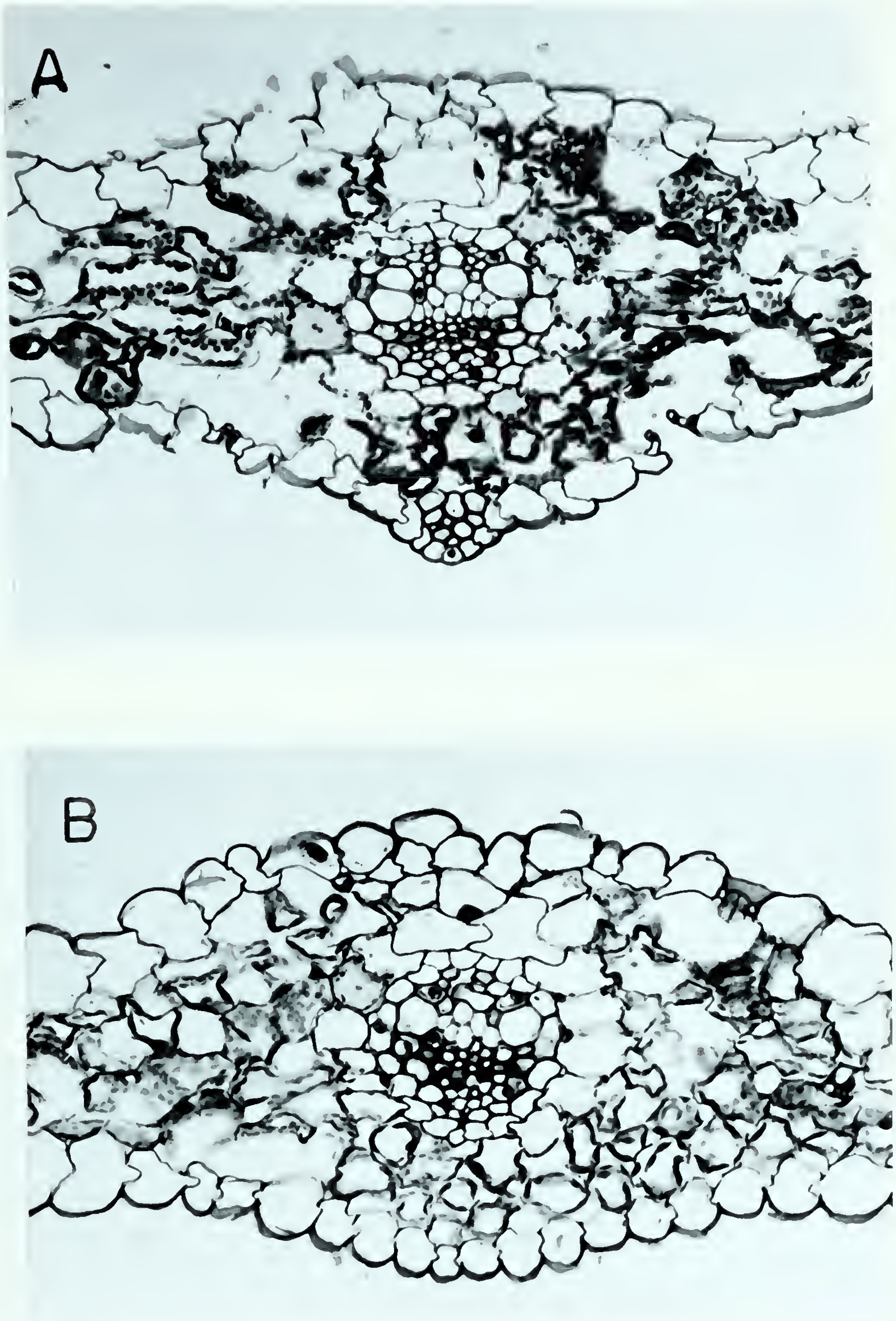
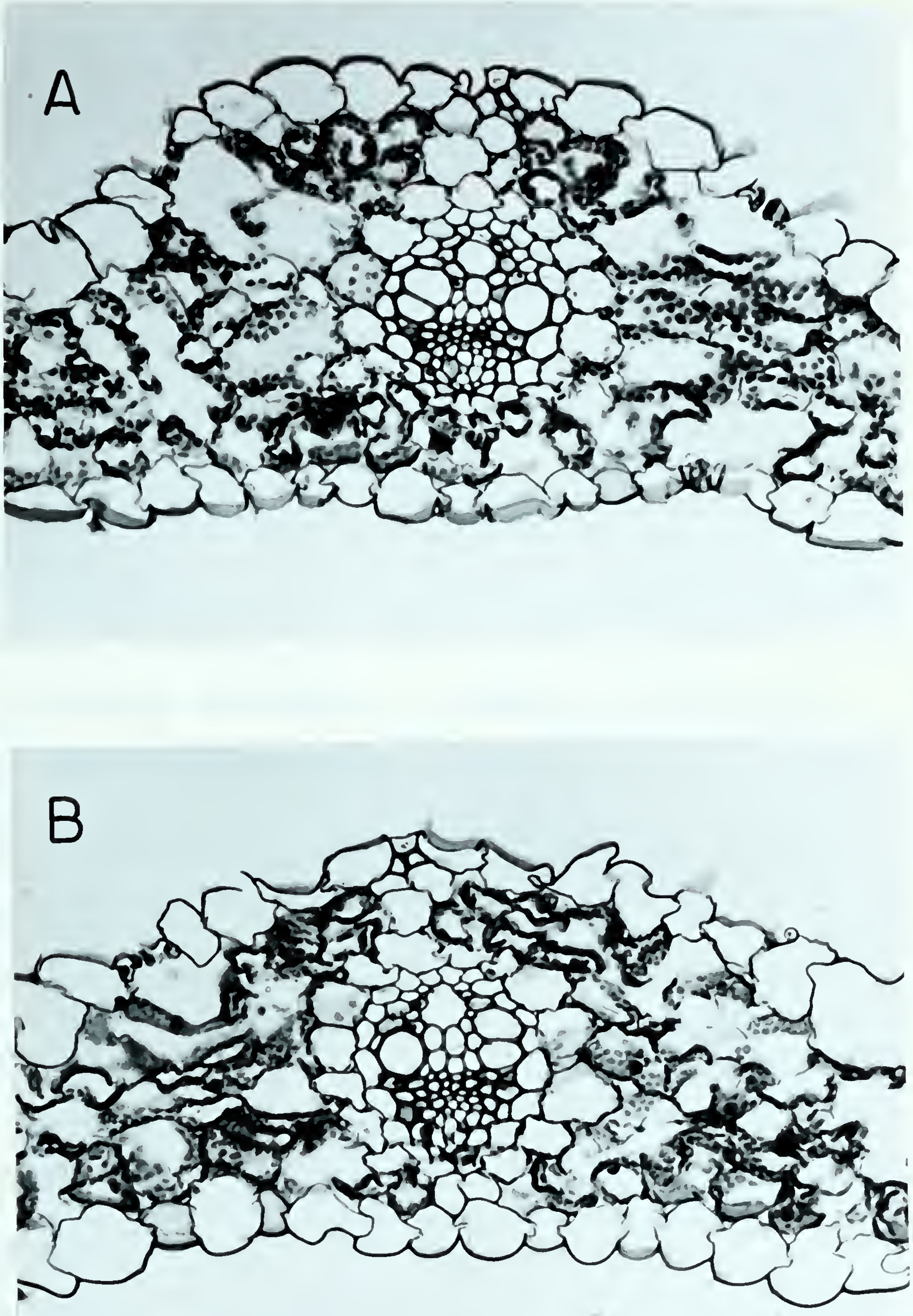

A
B

Figure 6. Photomicrographs of transverse sections cut through the mid-vein region of 11-day primary leaves of Gateway barley fixed in Rawlin's solution. A - normal; B - mutant. Mag. x 270.


A
B

in shape, though some are still somewhat flattened. It should be added that these distinguishing characteristics are not as clear in the photographs as they were in the original material.

Figures 7 and 8 show photomicrographs of living cells of normal and mutant leaves in sucrose solution. Figure 7 shows chloroplasts of normal and mutant cells at 2 and at 3 days after emergence of the seedling. The normal plastids (A and B) can be seen to contain grana, which often appear to lie in rows (arrows). The plastids are circular in surface view and lens-shaped in side view. They are approximately 5 u long and 3 u thick. The plastids of the mutant leaf cells at the same stage (C and D) are smaller (3 u long, 1.5 u thick) than the normal and they are less distinct in outline. It proved to be impossible to obtain pictures with better resolution than those shown and the vagueness of outline is a real phenomenon, also characteristic of etiolated plastids (Mego and Jagendorf, 1961). No grana or internal structure can be seen. The chloroplasts are flattened in shape in side view and some are rather irregular in outline, particularly at the 2-day stage. Figure 8A shows normal chloroplasts in leaf cells 10 days after emergence of the seedling. The plastids have not altered in size or shape from the 2-day stage. They can be seen to contain grana, but the granular texture is finer than that of young plastids. Figures 8B and 8C show the mutant chloroplasts at 10 days. In surface view they

Figure 7. Photomicrographs of living leaf cells from 2- and 3-day barley seedlings. Cells suspended in 0.75M sucrose solution. A - 2-day normal; B - 3-day normal; C - 2-day mutant; D - 3-day mutant. Mag. x 1570.

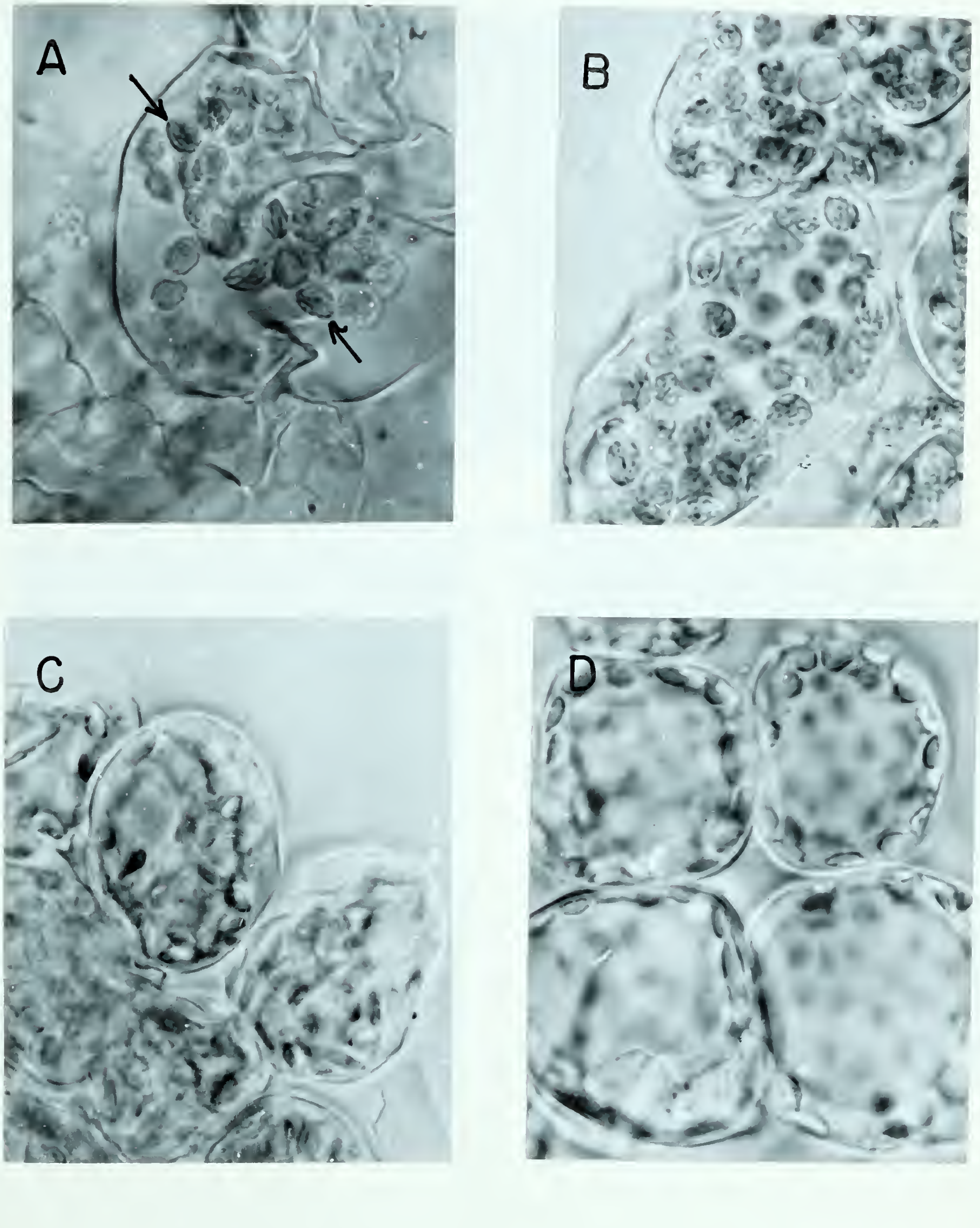
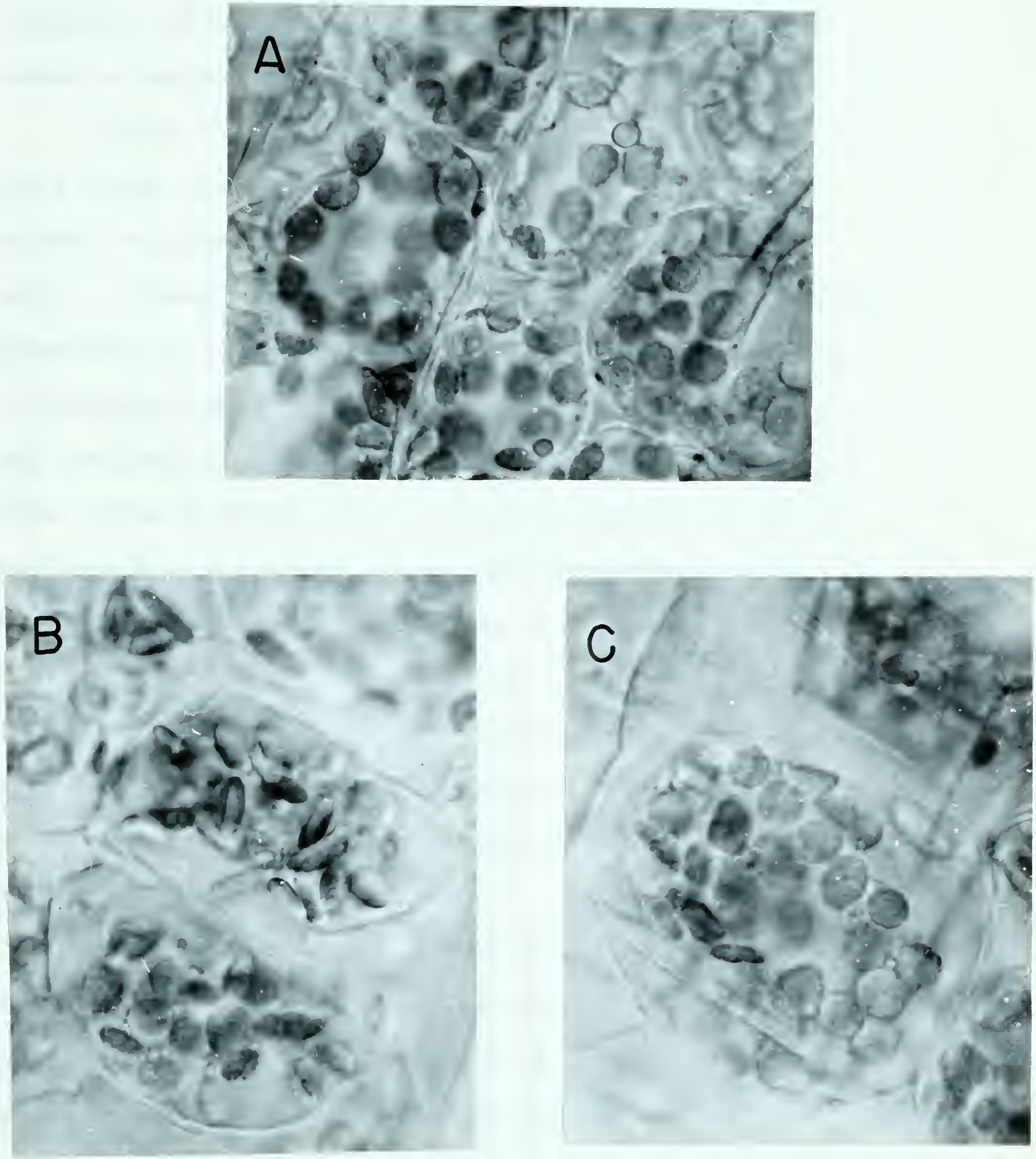

A
B
C
D

Figure 8. Photomicrographs of living cells from a 10-day barley primary leaf. Cells suspended in 0.75M sucrose solution. A - normal; B and C - mutant. Mag. x 1570.


A
B
C

resemble the normal plastids, i.e. they are distinct, circular (5 u in diameter) and finely granulated. In side view, however, they are flattened (only about 2 u thick). Thus diameter and granulation, if not thickness, of the mutant plastids have recovered to near-normal appearance with greening.

Figure 9 is an electron micrograph of a young plastid from a normal leaf at 2 days after emergence. The plastid is enclosed by a membrane (PM) which in places can be seen to be double. Internally there is a granular stroma (S) and a lamellar system which is still at an early stage of development. Stroma - or intergrana-lamellae are present (L) and small grana (Gr) appear throughout the plastid. The stroma contains several osmiophilic bodies or globuli (Gl). Very small granules can also be seen throughout the stroma. These could be ribosomes, since they look very much like the ribosomes of cytoplasm and such particles are now known to exist in chloroplasts (Lyttleton, 1962). Figure 10A shows another plastid from a 2-day normal leaf. This plastid is at an even earlier stage of development with several layers of lamellae but only rudimentary grana regions.

Figures 10B to 13 are electron micrographs of mutant plastids from 2-day leaf tissue. The mutant plastids are surrounded by a membrane which appears to be double (10B and 11A, arrows). However, they have a characteristically more irregular

Figure 9. Electron micrograph of a chloroplast from 2-day normal barley leaf tissue. Gl - globuli; Gr - granum; L - stroma - lamellae; PM - plastid membrane; S - Stroma. Mag. x 70,000.

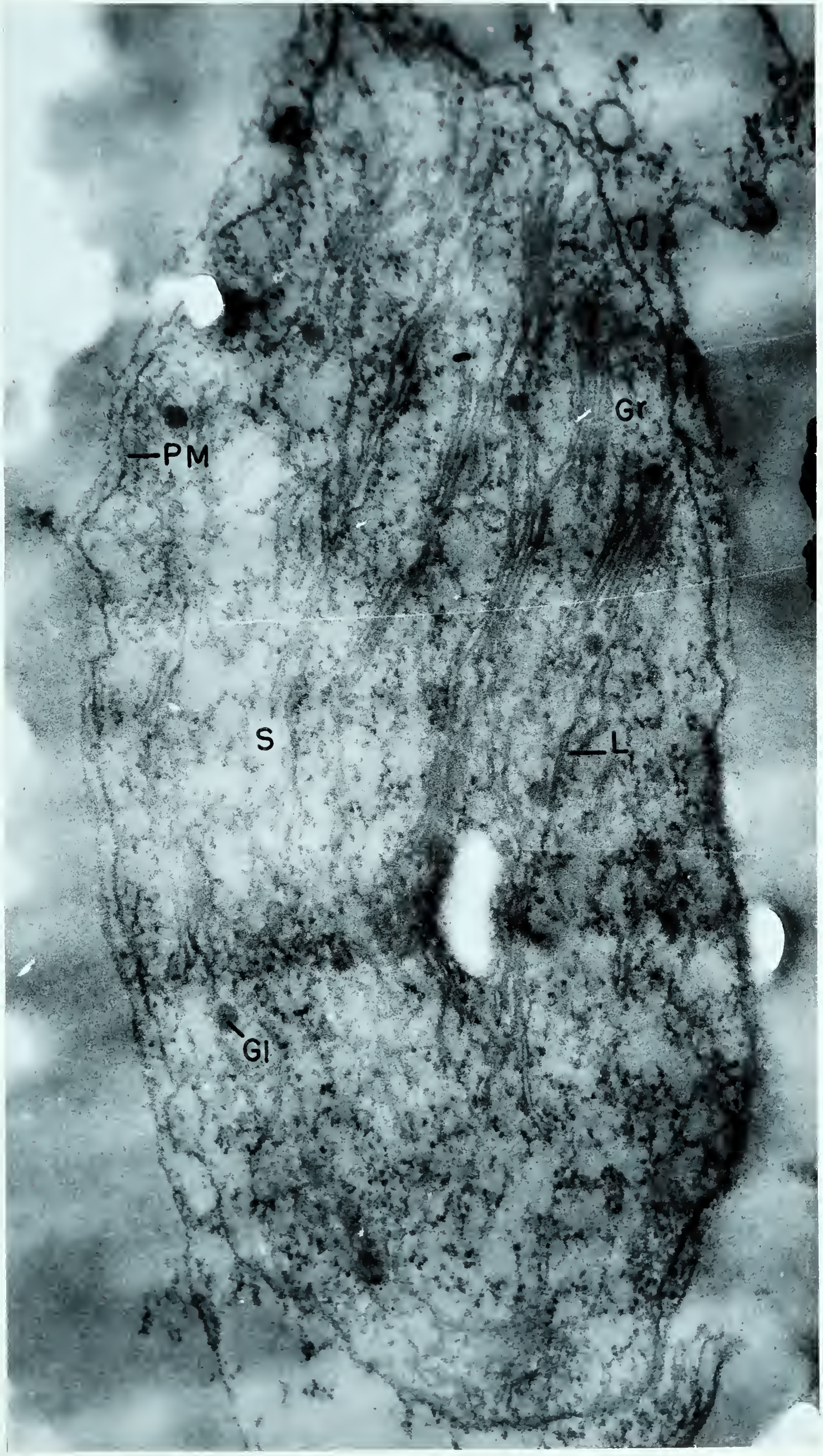
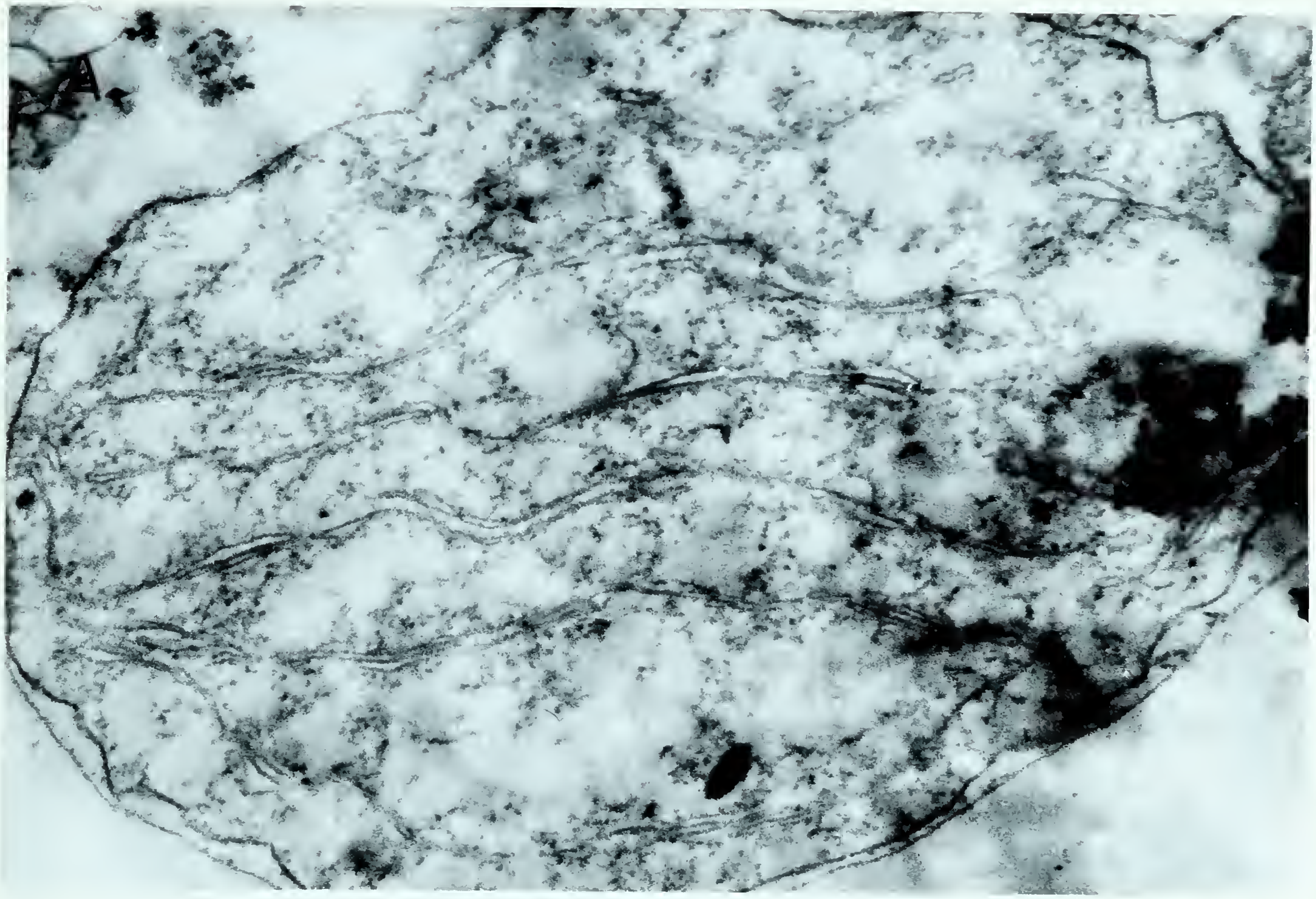

PM
Gr
S
L
Gl

Figure 10A. Electron micrograph of a chloroplast from 2-day normal barley leaf tissue. Mag. x 51,000.

Figure 10B. Electron micrograph of a chloroplast from 2-day mutant barley leaf tissue. PM - Plastid membrane; V - vesicle. Mag. x 57,000.


A

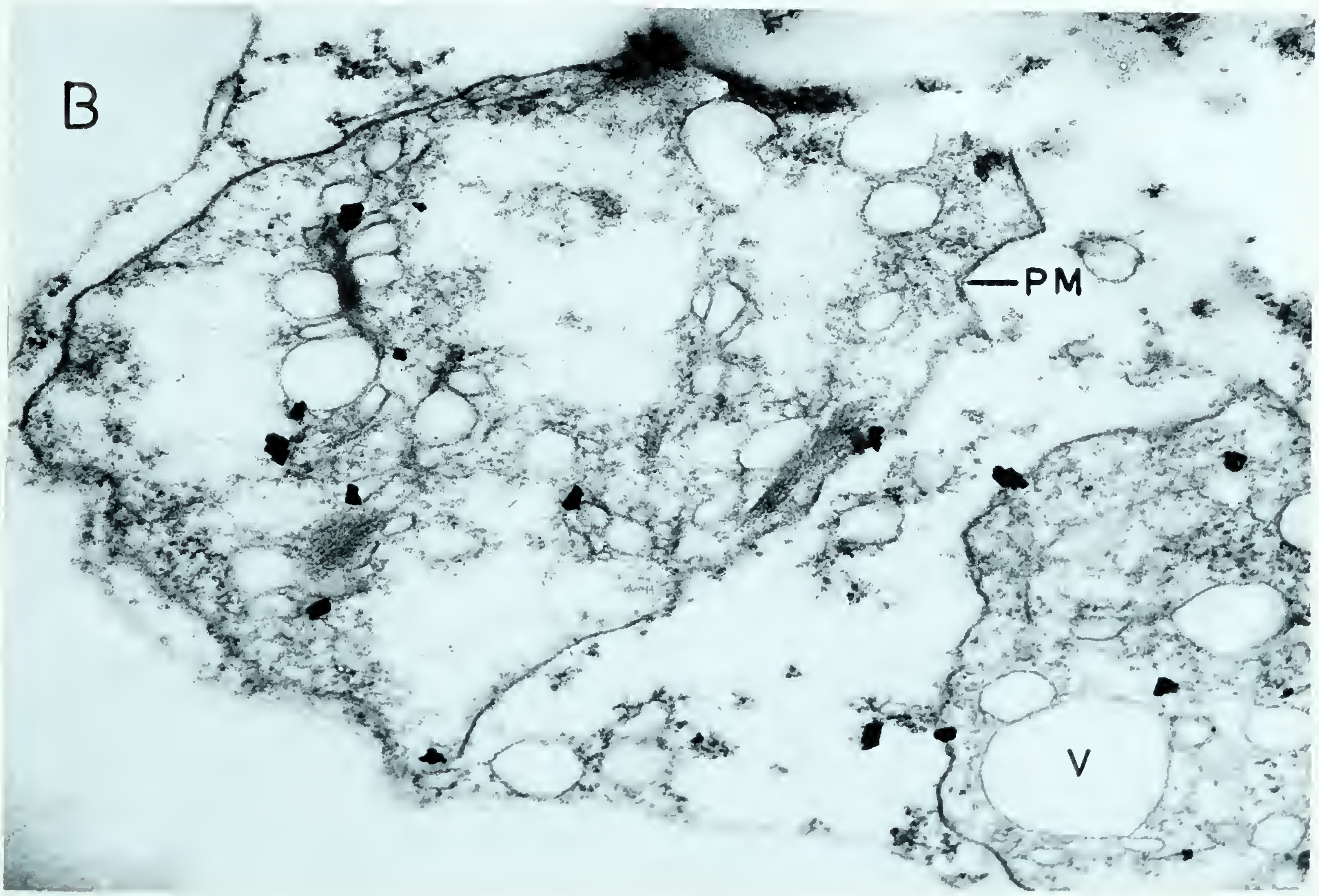
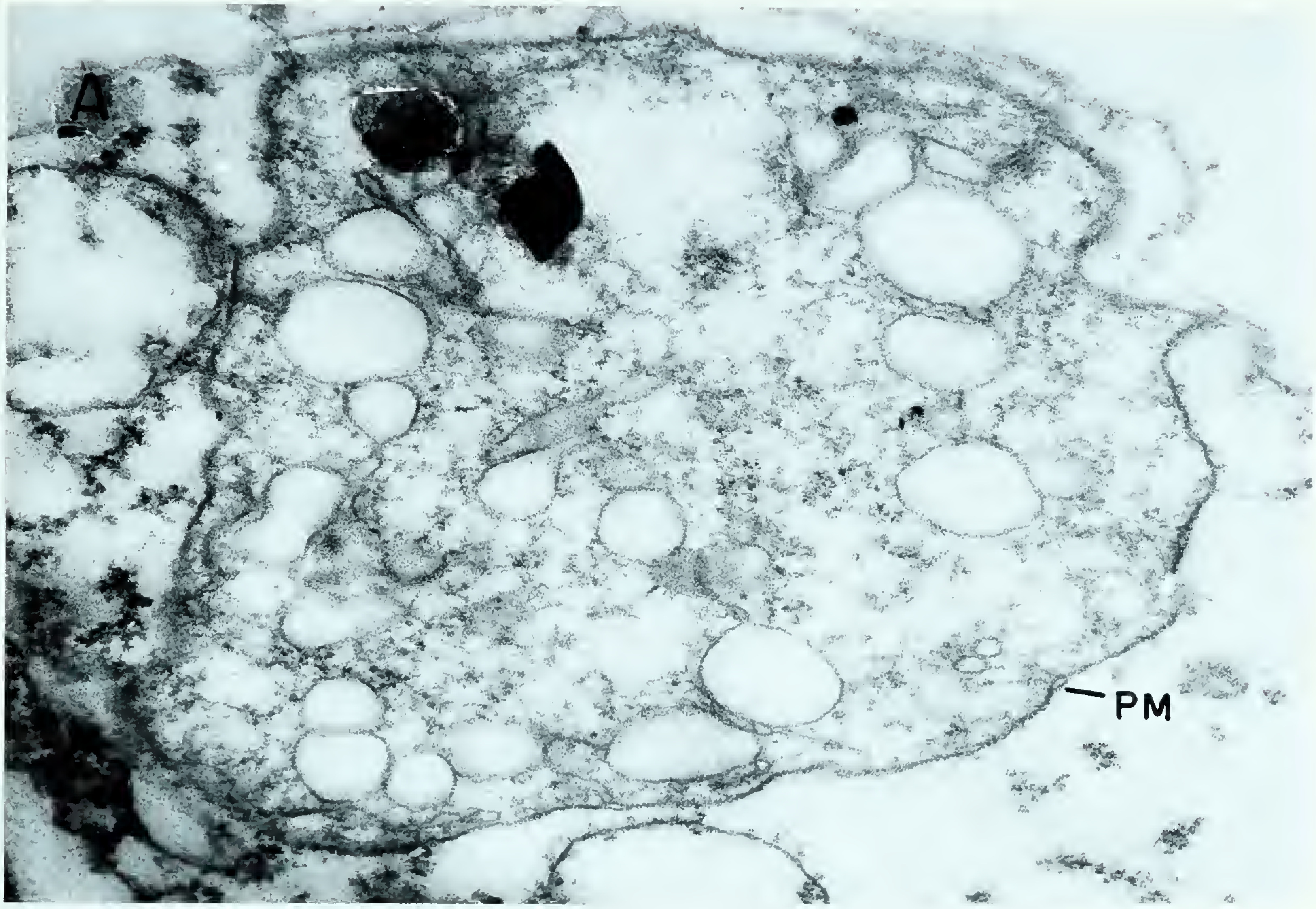

B
PM
V

Figure 11 (A and B). Electron micrographs of chloroplasts from 2-day mutant barley leaf tissue. PM - plastid membrane. Mag. x 64,000.


A
PM

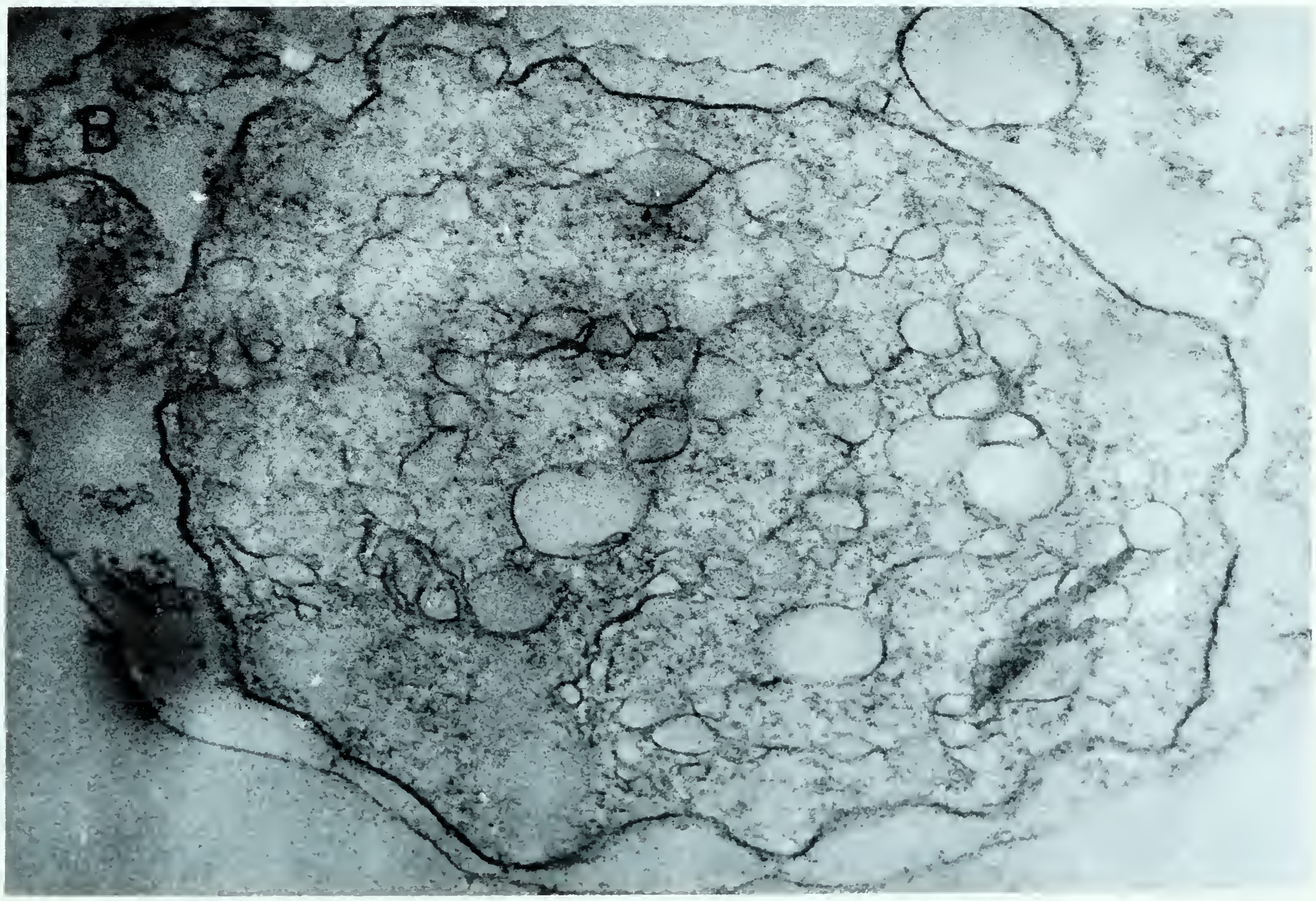

B

Figure 12. Electron micrographs of chloroplasts from 2-day mutant barley leaf tissue. LS - layered structure. Mag. x 75,000.

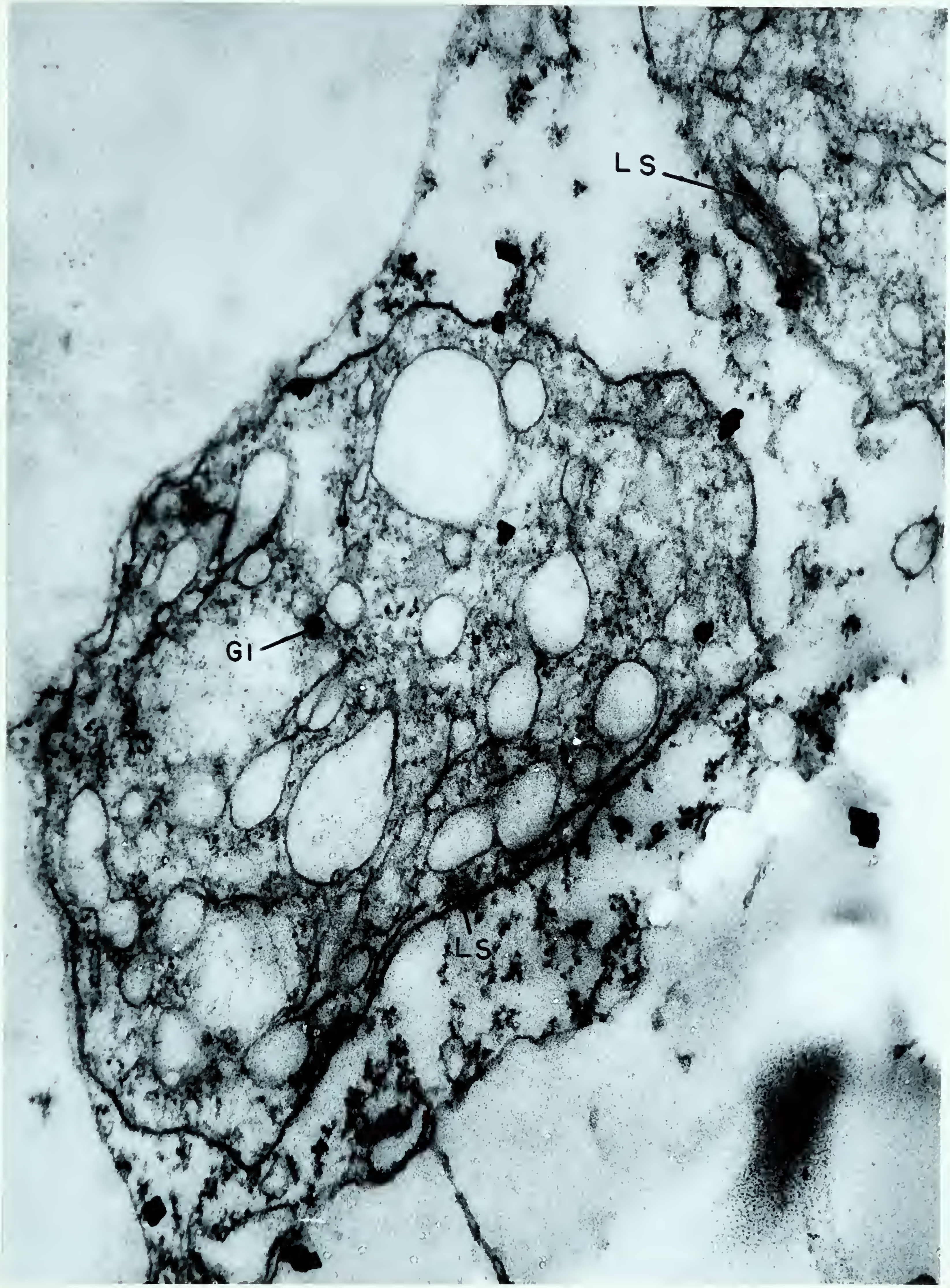
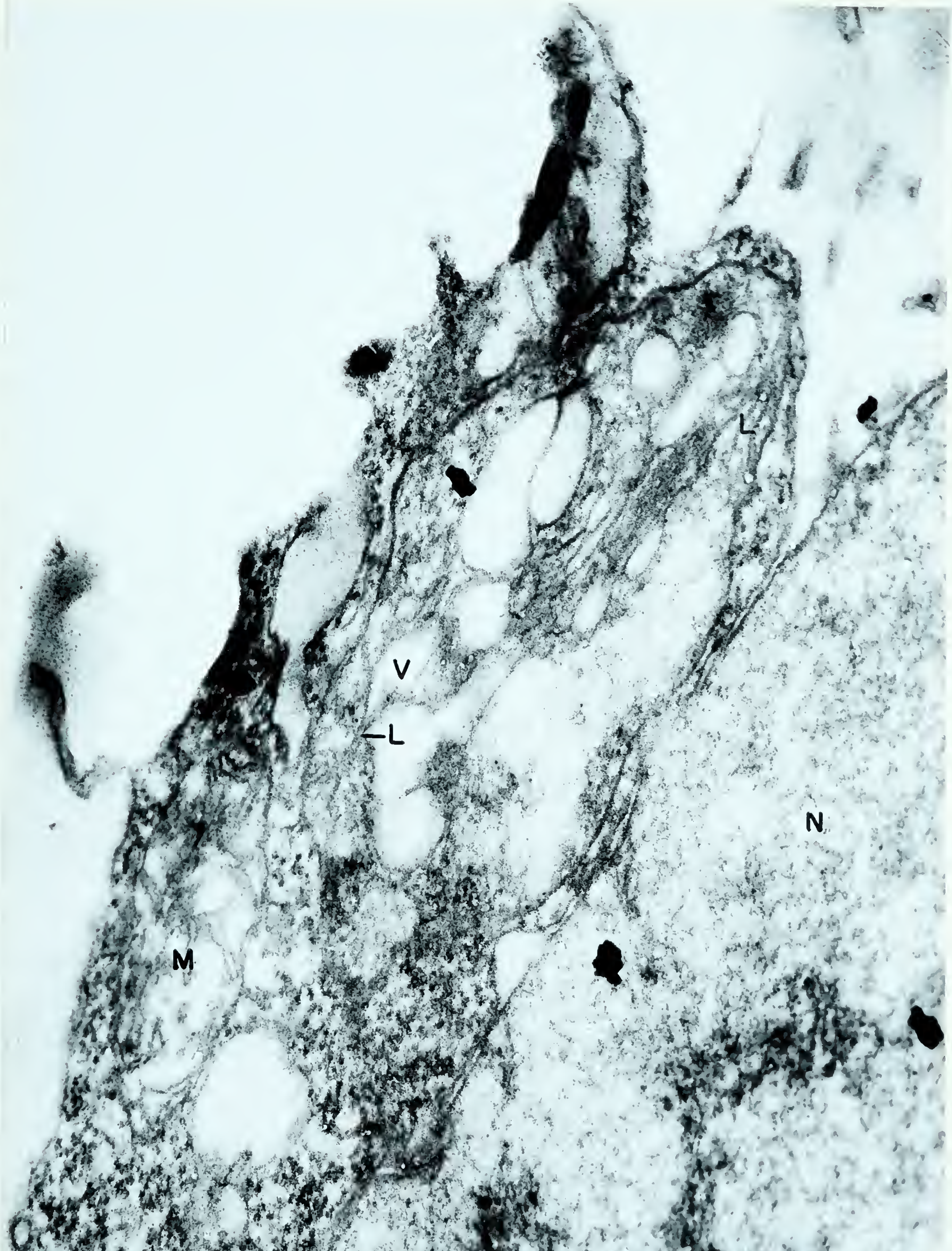

LS
GI
LS

Figure 13. Electron micrograph of a chloroplast, a mitochondrion(M) and a portion of a nucleus (N) from 2-day mutant barley leaf tissue. L - stroma - lamellae; V - vesicle. Mag. x 90,000.


L
V
L
N
M

shape than normal plastids and they are considerably smaller. Internally they contain a granular stroma and occasional globuli (Fig. 12) but there is seldom any differentiation into typical lamellae and grana. Occasionally fragments of what appear to be layered structures (LS) are visible (Fig. 12) and in one case (Fig. 13) the plastid quite definitely contains lamellae. These seem to be attached to, or arising from, vesicles (V). In the majority of plastids the empty vesicles are the only visible internal structures. They are often associated in chains (Fig. 11B). In a few cases they are compartmented and may be young grana which have swollen during fixation (see later discussion of 4-day material). In general, the plastids correspond to the amoeboid stage in plastid development described by von Wettstein (1958) except that they have unusually large vesicles.

Figures 14 to 16 are electron micrographs of normal and mutant chloroplasts from 4-day leaves. The normal plastid at this age (Fig. 14) differs little from the 2-day plastid (Fig. 9) except that the grana have developed a few more layers. They are well formed with distinct compartments which contain no granules and appear quite empty. The mutant plastids at 4 days (Fig. 15) contain what appear to be empty but many-compartmented vesicles. These resemble the swollen grana seen in electron micrographs of isolated grana and chloroplasts (cf. Rabinowitch 1956, Wehrmeyer,

Figure 14. Electron micrograph of a chloroplast from 4-day normal barley leaf tissue. Gr - granum; L - stroma-lamellae. Mag. x 78,000.

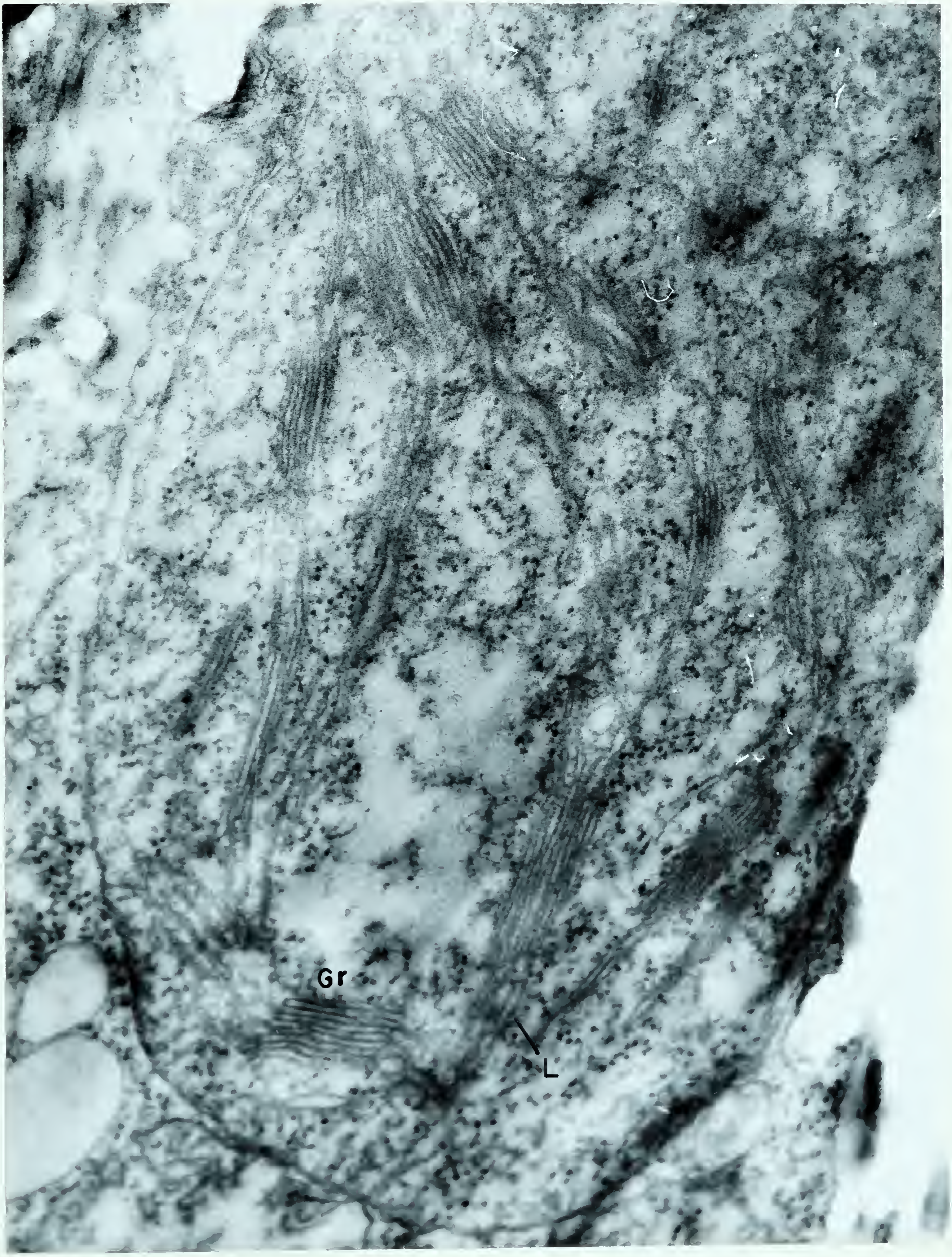

Gr
L

Figure 15. Electron micrograph of two chloroplasts from 4-day mutant barley leaf tissue. Gl - globuli; Gr - granum. Mag. x 40,000.

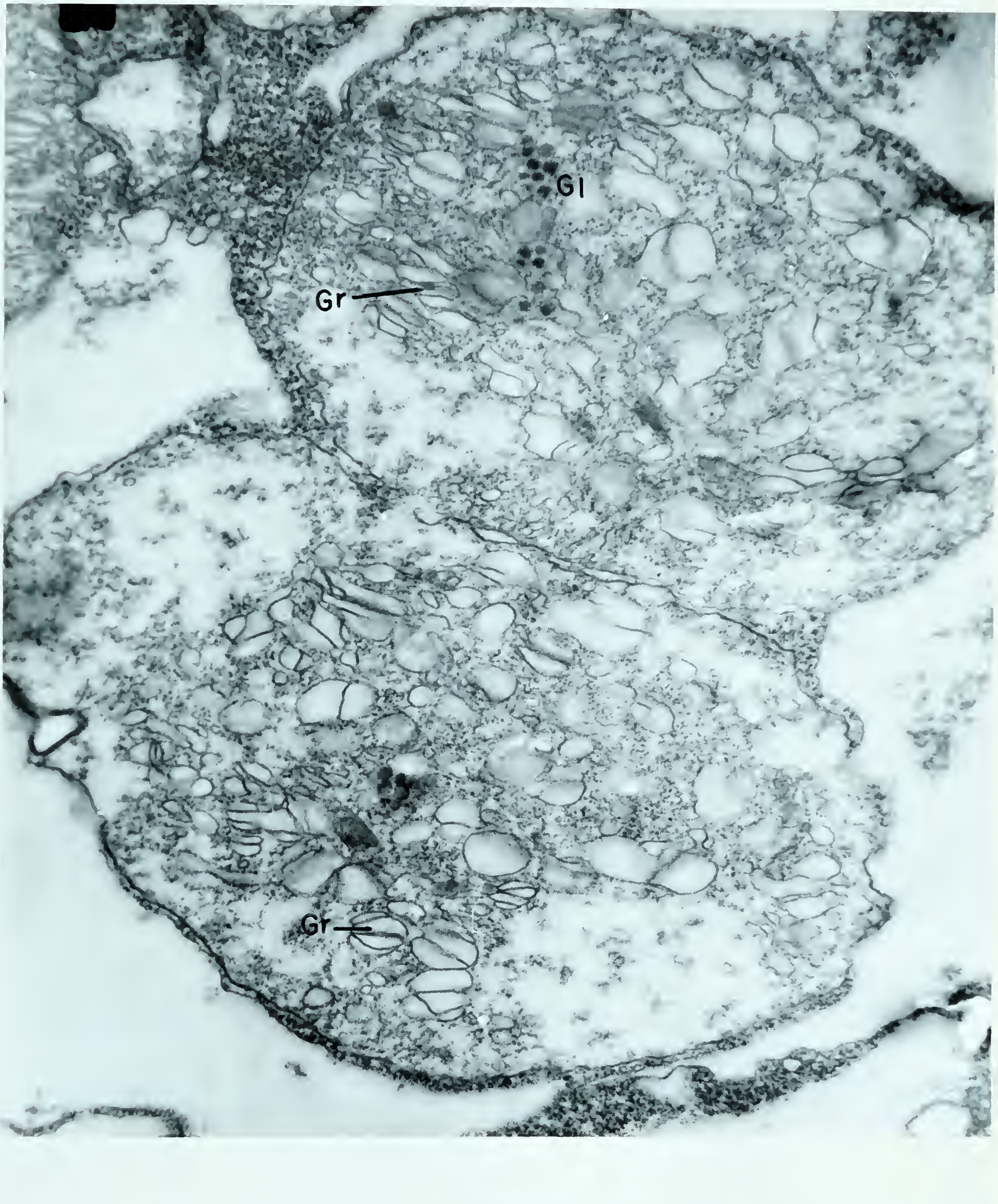
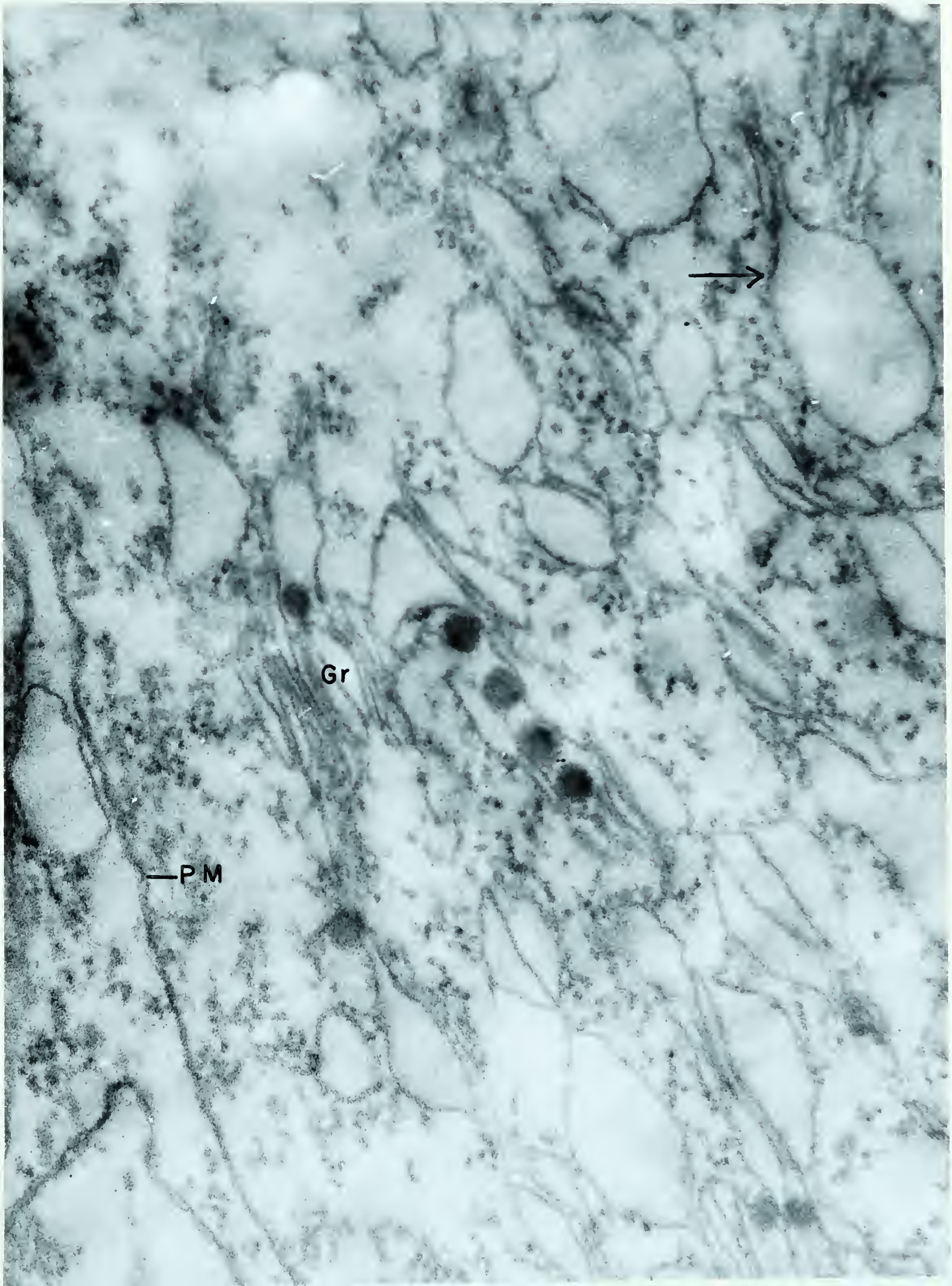

Gl
Gr
Gr

Figure 16. Electron micrograph of a portion of a chloroplast from a 4-day mutant barley leaf tissue. Gr - granum; PM - plastid membrane. Mag. x 97,000.


Gr
PM

1962). In cases where the vesicles are not compartmented (Fig. 16) they appear (arrow) to be swollen lamellae, again similar to those of isolated chloroplasts (Wehmeyer, 1962).

Figures 17 to 20 are electron micrographs of normal and mutant primary leaf tissue taken 12 days after emergence. These specimens have poorer resolution than the younger material, perhaps because of poorer penetration of fixative in older tissues. Nevertheless, it can be seen that the grana and lamellae in both normal and mutant chloroplasts are well developed. The double nature of the lamellae is apparent in the more highly magnified normal plastid (Fig. 17 at L). There are many globuli present, particularly in the mutant plastids (Figs. 18B, 19, 20). It is clear that the internal structure of mutant plastids is quite normal at this stage.

Figure 17. Electron micrograph of a portion of a chloroplast from 12-day normal primary leaf tissue. Gl - globuli; Gr - granum; L - stroma-lamellae. Mag. x 115,000.

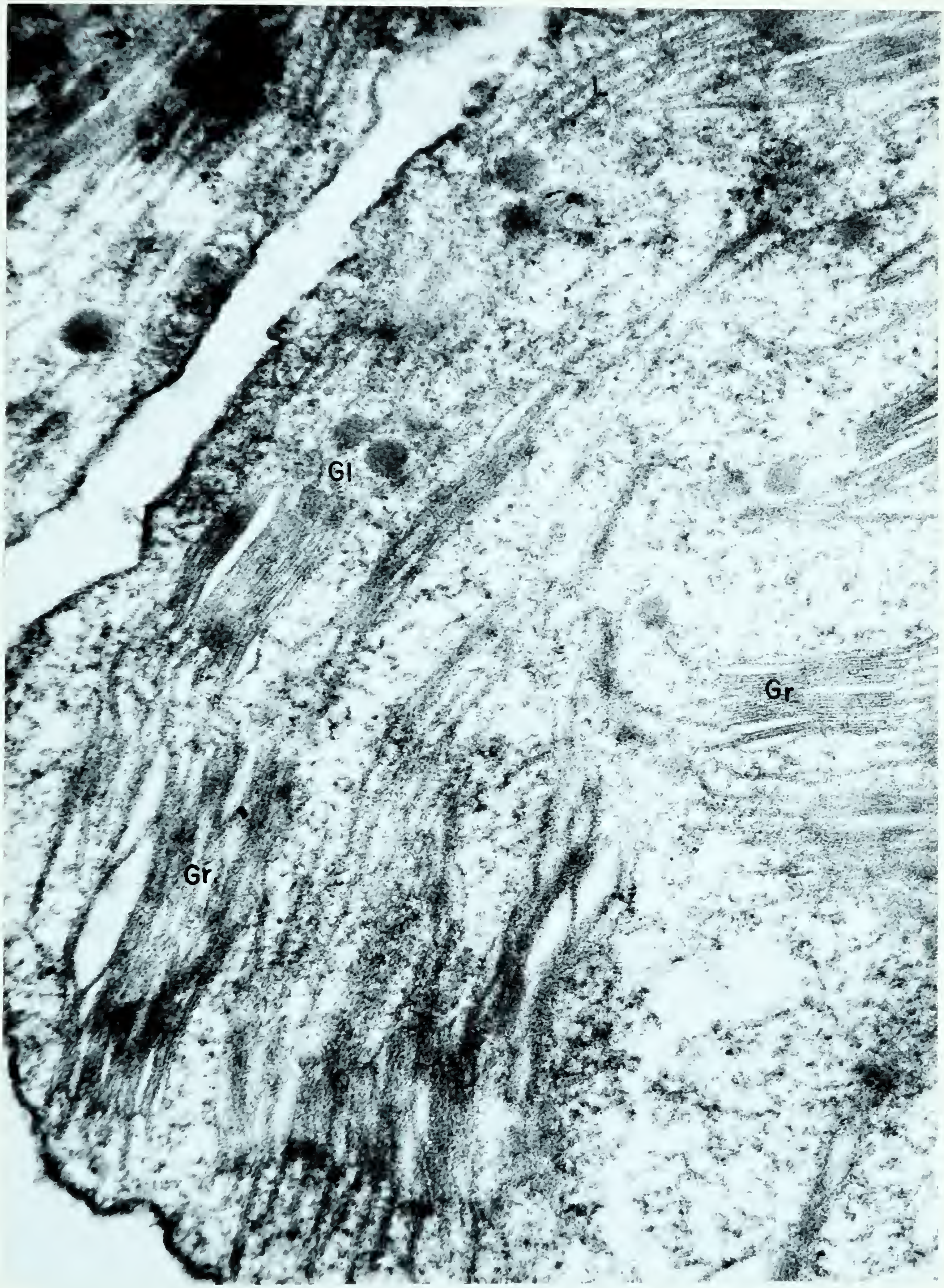

Gl
Gr
Gr

Figure 18A. Electron micrograph of a chloroplast from 12-day normal primary leaf tissue. Mag. x 35,000.

Figure 18B. Electron micrograph of a portion of a chloroplast from 12-day mutant primary leaf tissue. Mag. x 65,000.

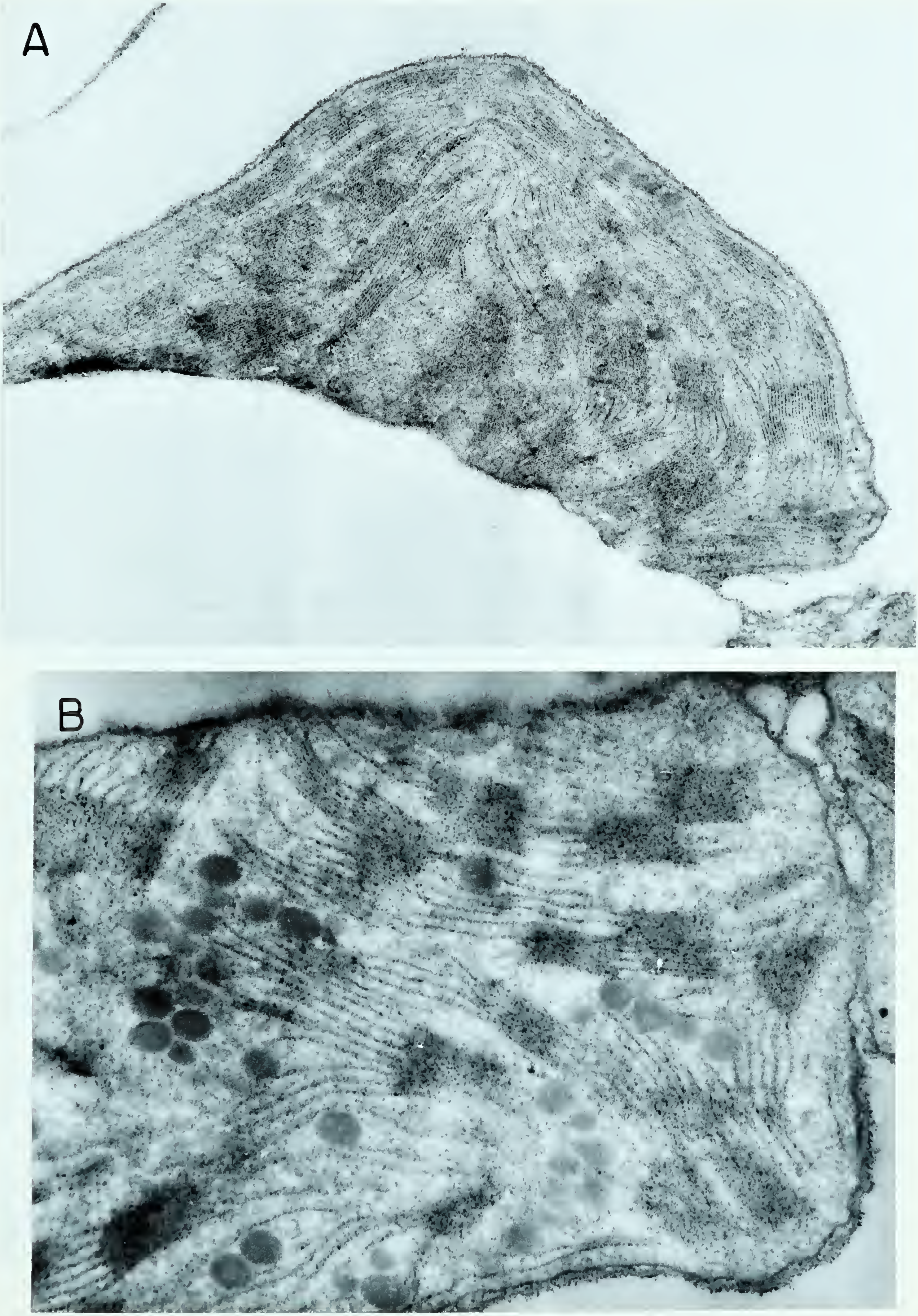

A
B

Figure 19. Electron micrograph of a portion of a chloroplast from 12-day mutant primary leaf tissue. Gl - globuli; Gr - granum; L - stroma-lamellae. Mag. x 68,000.

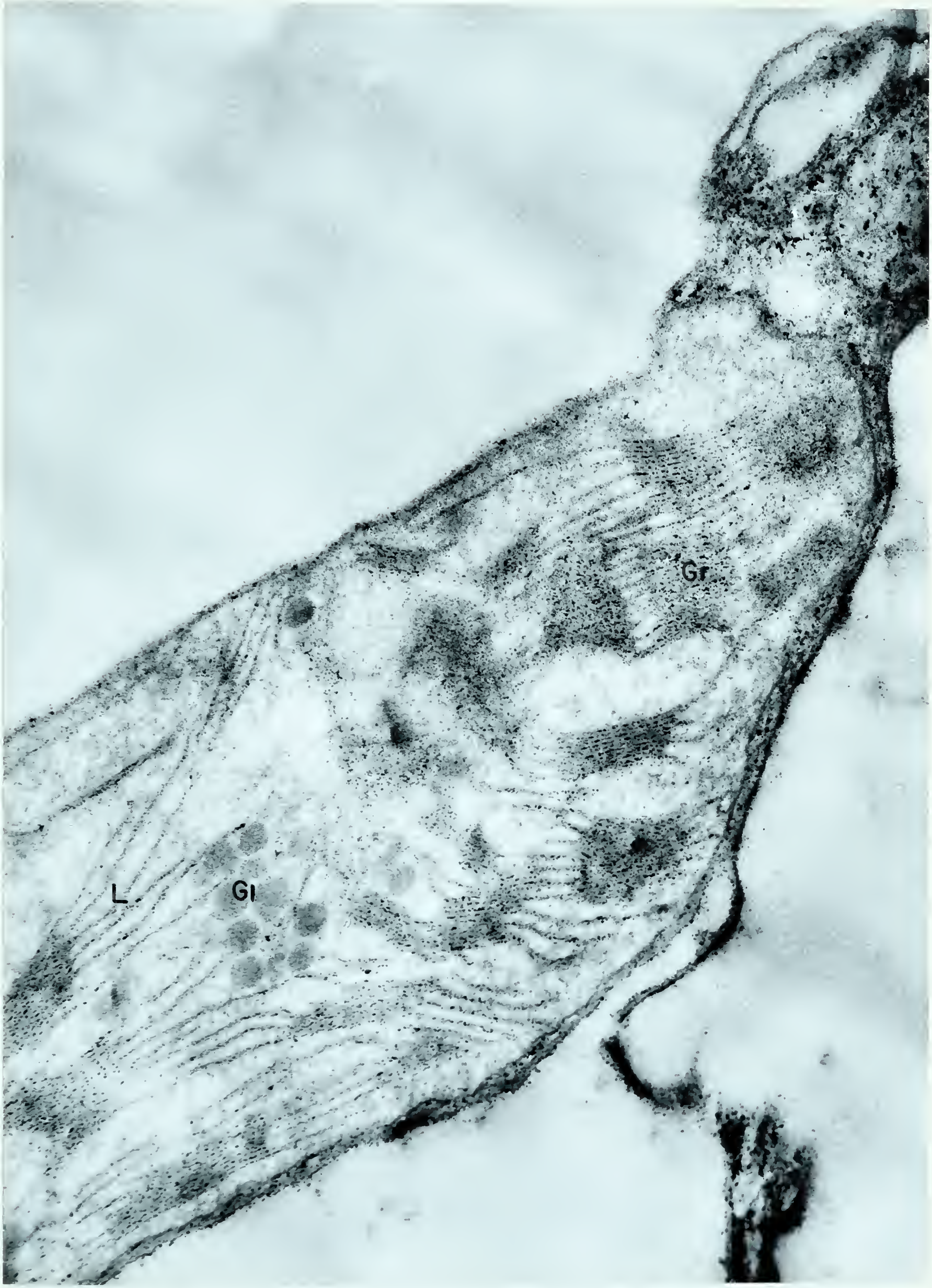
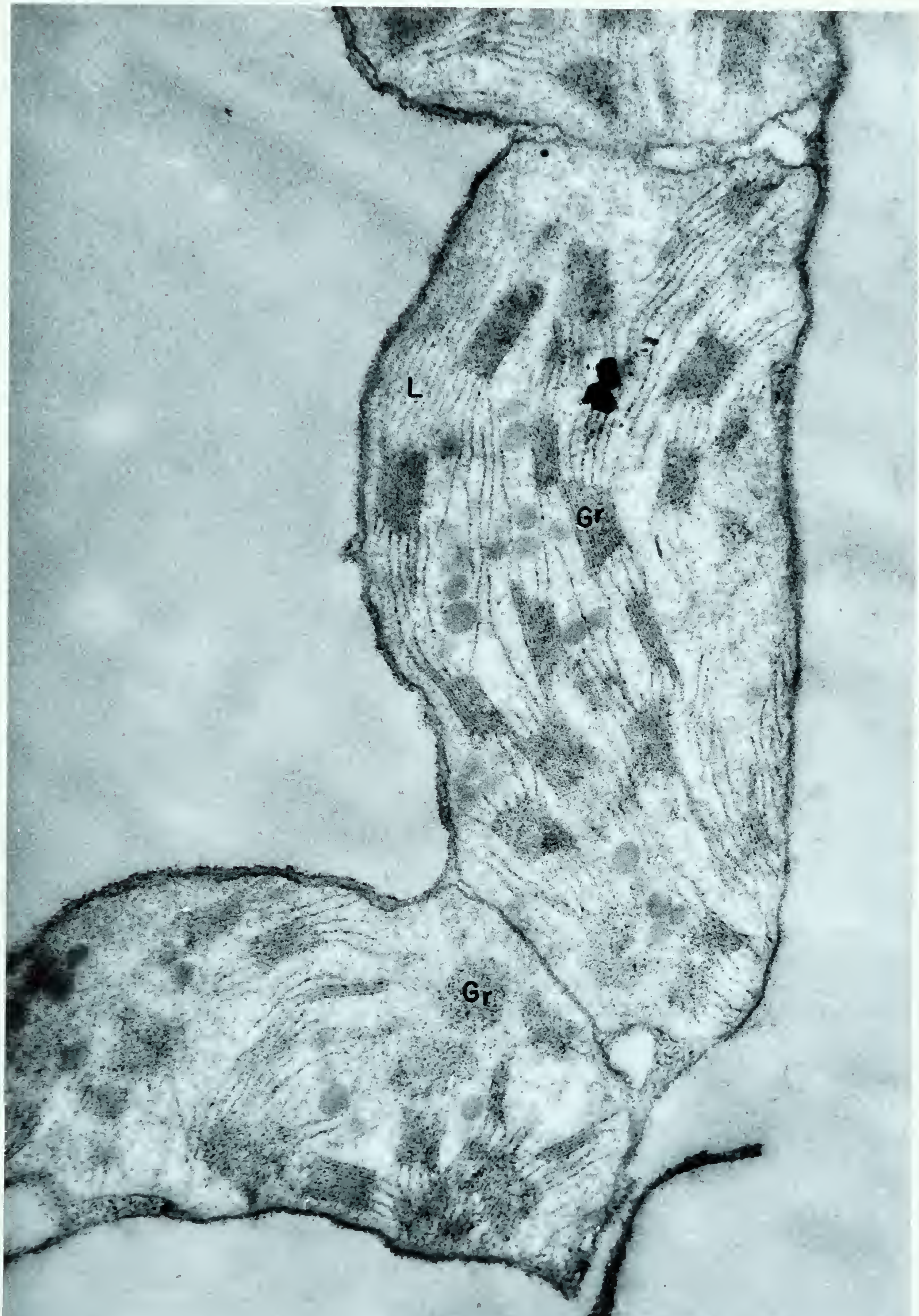

Gr
L
Gl

Figure 20. Electron micrograph of chloroplasts from 12-day mutant primary leaf tissue. Gr - granum; L - stroma-lamellae. Mag. x 44,000.


L
Gr
Gr

3. Fractionation Experiments.

Nitrogen determinations were made on the following three fractions of leaf homogenates:

Fraction 1 - particles: - nuclei, chloroplasts, mitochondria;

Fraction 2 - acid-insoluble precipitate: - microsomes, proteins and nucleic acids;

Fraction 3 - acid-soluble supernatant: - free amino acids, acid-soluble polypeptides, nucleotides.

In one experiment estimations were made at 4, 6 and 12 days after emergence of normal and mutant seedlings grown in the greenhouse. The experiment was repeated using 5- and 10-day seedlings grown in the growth chamber. Results for the two experiments are shown in Tables IV and V respectively.

The total nitrogen per gram fresh weight of the three fractions was higher in the mutant than in the normal at the 4- and 5-day stages. This was due to the very high nitrogen content of the acid-soluble fraction in the mutant since the other fractions were actually somewhat lower in the mutant than in the normal.

By 6 days the total nitrogen level of the mutant had dropped markedly, with the result that the mutant's nitrogen at this stage was lower than that of the normal. The decrease was due to a 40% loss of acid-soluble nitrogen during this 2-day

TABLE IV

DISTRIBUTION OF NITROGEN IN LEAF FRACTIONS

OF 4-, 6- AND 12-DAY

NORMAL AND MUTANT BARLEY SEEDLINGS

Chlorophyll determinations are given below

to show stage of greening in these samples.

Fraction	mg.N/g.fr.wt. *					
	4-Day		6-Day		12-Day	
	N	M	N	M	N	M
Particles	1.50 ±.03	1.38 ±.01	1.76 ±.02	1.31 ±.01	1.70 ±.02	1.48 ±.01
Acid-insoluble	2.10 ±.04	1.70 ±.03	2.29 ±.07	1.74 ±.02	1.83 ±.03	1.34 ±.01
Acid-soluble	1.30 ±.03	2.37 ±.04	1.09 ±.04	1.42 ±.02	0.90 ±.02	1.05 ±.03
Total	4.90 ±.05	5.45 ±.07	5.14 ±.07	4.47 ±.04	4.43 ±.04	3.87 ±.04
	Corresponding chlorophyll contents mg.C/g.fr.wt.					
	0.98	0.18	1.08	0.46	1.60	1.16

* Values are averages of 4 determinations with the standard error given below each.

TABLE V

DISTRIBUTION OF NITROGEN IN LEAF FRACTIONS

OF 5- AND 10-DAY

NORMAL AND MUTANT BARLEY SEEDLINGS

Chlorophyll determinations are given below

to show stage of greening in these samples.

Fraction	mg.N/g.fr.wt. *			
	5-Day		10-Day	
	N	M	N	M
Particles	1.56 ±.01	1.47 ±.01	1.73 ±.01	1.53 ±.01
Acid-insoluble	1.71 ±.00	1.61 ±.04	1.77 ±.00	1.66 ±.01
Acid-soluble	0.78 ±.03	1.74 ±.07	0.40 ±.01	0.59 ±.02
Total	4.05 ±.03	4.82 ±.11	3.90 ±.03	3.78 ±.02
	Corresponding chlorophyll contents mg.C/g.fr.wt.			
	1.11	0.30	1.95	1.02

* Values are averages of 4 determinations with the standard error given below each.

period. The particulate and acid-insoluble fractions were not much altered in nitrogen content. The normal showed a small (16%) loss in acid-soluble nitrogen, but small increases in both particulate and acid-insoluble nitrogen resulted in a net gain in total nitrogen over the 2-day period.

By the 10- and 12-day stages the nitrogen balance had altered again. The same downward trend was apparent in the acid-soluble nitrogen levels of both normal and mutant. Acid-insoluble nitrogen and particulate nitrogen levels remained essentially the same in both normal and mutant.

Thus the major difference between mutant and normal in nitrogen content is the high level of acid-soluble nitrogen in the young mutant. This fraction was therefore examined in more detail in order to specify the components responsible.

The free amino acid content of the normal and mutant at 4- and 12-days is shown in Table VI, expressed as micromoles per gram fresh weight, and in Table VII, expressed as a percent of the total free amino acids. For comparison, the amino acid contents of the 5-day normal and mutant are given in Table ii of the Appendix. The results show that the young mutant seedling had a much higher content than the normal (2 to 6 times greater) of almost all of the 18 amino acids measured. The exceptions were glutamic acid and glycine, the levels of which were higher in the normal than the mutant (see results, however, for 5-day

TABLE VI

FREE AMINO ACID CONCENTRATIONS IN ACID-SOLUBLE FRACTIONS

OF 4- AND 12-DAY NORMAL AND MUTANT LEAVES

Plants grown in greenhouse.

Amino Acid	um.a.a./g.fr.wt.			
	4-Day		12-Day	
	N	M	N	M
Lysine	0.10	0.41	0.05	0.08
Histidine	0.14	1.08	0.01	0.01
Ammonia	1.24	4.78	0.71	1.16
Arginine	trace	0.38	trace	0.00
Aspartic acid	1.58	3.22	1.27	1.31
Threonine	0.52	0.89	0.42	0.52
Serine	4.56	26.05	1.08	1.25
Glutamic acid	3.30	2.60	2.74	2.77
Proline	0.00	0.00	0.00	trace
Glycine	0.91	0.77	0.21	0.56
Alanine	1.48	2.72	1.03	1.24
Cystine	0.00	trace	0.00	0.00
Valine	0.27	1.55	0.05	0.04
Methionine	trace	0.06	0.00	0.01
Isoleucine	0.09	0.66	0.02	0.02
Leucine	0.10	0.69	0.03	0.03
Tyrosine	0.05	0.08	0.00	trace
Phenylalanine	0.07	0.06	0.01	0.02
Total	14.41	46.00	7.63	9.02

TABLE VII

FREE AMINO ACID CONCENTRATIONS RELATIVE TO TOTAL AMINO ACID CONCENTRATIONS IN ACID-SOLUBLE FRACTIONS

(See TABLE VI).

Amino Acid	$\frac{\text{um.a.a./g.fr.wt.}}{\text{total um.a.a./g.fr.wt.}}$ x 100			
	4-Day		12-Day	
	N	M	N	M
Lysine	0.7	0.9	0.6	0.9
Histidine	1.0	2.4	0.1	0.2
Ammonia	8.6	10.4	9.3	12.9
Arginine	0.0	0.8	0.0	0.0
Aspartic acid	11.0	7.0	16.7	14.6
Threonine	3.6	1.9	5.5	5.8
Serine	31.7	56.6	14.2	13.9
Glutamic acid	22.9	5.6	36.0	30.8
Proline	0.0	0.0	0.0	0.0
Glycine	6.3	1.7	2.8	6.2
Alanine	10.3	5.9	13.6	13.8
Cystine	0.0	0.0	0.0	0.0
Valine	1.8	3.4	0.6	0.4
Methionine	0.0	0.1	0.0	0.1
Isoleucine	0.6	1.4	0.2	0.2
Leucine	0.7	1.5	0.3	0.3
Tyrosine	0.3	0.2	0.0	0.0
Phenylalanine	0.5	0.1	0.2	0.3

plants). Tyrosine and phenylalanine were present at approximately the same levels in normal and mutant but they occurred in very small amounts. The case of serine is particularly interesting. In the normal seedling it was present in higher concentration than any other amino acid, representing nearly one third of the total amino acid content (32% in 4-day, 28% in 5-day seedlings). In the mutant seedlings its concentration was 6 times as high as in the normal and accounted for more than one half the amino acid content (57% in 4-day, 55% in 5-day seedlings).

At 12 days the amino acid levels of normal and mutant were lower than in young tissues and remarkably close to one another. By this stage serine, for example, accounted for 14% of the total amino acid content in both normal and mutant. Obviously the free amino acid balance in the mutant, distorted in young tissue, was restored nearly to normal by the time the leaves had become fully green.

The proportion of total nitrogen in the acid-soluble fractions (Table IV) which was accounted for by free amino acids, ribonucleotides and unknown remaining substances is shown in Table VIII. As already noted (Table VI), free amino acid levels were very high in the young mutant and declined to near normal levels by 12 days. Free ribonucleotide levels were, in contrast, low in the young mutant and rose to near normal levels by 12 days.

TABLE VIII

NITROGEN CONTENT OF FREE AMINO ACIDS, RIBONUCLEOTIDES AND THE REMAINDER OF THE ACID-SOLUBLE FRACTION (TABLE IV) OF NORMAL AND MUTANT LEAVES

Fraction	4-Day		12-Day	
	N	M	N	M
Amino acids (mg.N/g.fr.wt.)	0.208	0.694	0.107	0.127
Ribonucleotides* (mg.N/g.fr.wt.)	0.450	0.195	0.465	0.375
Remainder (mg.N/g.fr.wt.)	0.640	1.483	0.323	0.552

* Measured as RNA equivalents and calculated assuming that RNA contains 15% N by weight (see Appendix, Table i).

The remaining soluble nitrogen accounted for a large part of the total nitrogen in young tissues, particularly in the mutant. The concentration of this nitrogen fell with age of tissue and the decrease was responsible for a large proportion of the decrease in nitrogen content of the greening mutant leaf (Table IV). It should be noted that the nitrogen level in this unknown fraction tended to adjust in the mutant towards that of the normal by 12 days.

DISCUSSION

The mutant of Gateway barley used in this investigation belongs to the viridis or pale-green class of chlorophyll mutants. The levels of chlorophylls a and b and of carotenoid pigment in relation to normal seedling levels fall within the range of concentrations given by Holm (1954) for the viridis mutants which he studied. The chloroplasts in the Gateway mutant are smaller than normal, which Holm states is true of viridis mutants in general. Moreover, the chloroplasts eventually develop normal internal structures, and this is also claimed to be true of at least some viridis mutants (von Wettstein, 1958). The ability of the Gateway mutant to become greener with age marks it as a "virescens" type of viridis mutant. To date, few microscopic investigations and no biochemical studies of such mutants have been reported in the literature. The results of studies of this type on the Gateway mutant have shown that it differs from normal Gateway barley in rate of pigment accumulation, in chloroplast development, and in nitrogen metabolism.

The mutant seedling at emergence is low in chlorophyll and carotenoid pigments. In medium light intensities and temperatures primary leaves of mutant seedlings become so green in a period of less than 2 weeks that they are visually almost indistinguishable from the normal. The recovery is not complete,

however, and pigment concentrations never reach levels higher than 80% of those in normal leaves. The overall increase in chlorophyll content from the 3rd day until maximum levels are reached is approximately the same for normal and mutant.(Fig. 3). The maximum rate of chlorophyll accumulation in the two is also similar. The difference between them is that the normal shoot is bright green at emergence, and accumulates chlorophyll at a rapid rate from the start, whereas the mutant is pale yellow at emergence and remains so for the first 3 to 4 days. There is, therefore, a definite lag period in the mutant before maximum rate of greening begins.

It is difficult simply by measuring chlorophyll accumulation to establish whether or not the lag period in the mutant is due to a lower rate of synthesis or a more rapid rate of breakdown of chlorophyll than in the normal. In albino plants that contain protochlorophyll, exposure to light results in conversion to chlorophyll a which, however, is unstable and slowly bleaches (Koski and Smith, 1951). Chlorophyll instability is presumably the reason for this particular type of albinism. In similar experiments with the Gateway mutant exposed to low light intensities, chlorophyll, once formed, was maintained (Fig. 4) suggesting that in this case, at least low levels of chlorophyll are resistant to bleaching. Indeed, the levels of chlorophyll in the young mutant grown in moderate light intensities are low but by no means

insignificant (Fig. 3). Nevertheless, some instability is indicated by the fact that high light intensities will cause bleaching. With respect to the rate of chlorophyll synthesis, the conversion of protochlorophyll to chlorophyll was very efficient in the etiolated mutant, but the rate of synthesis of protochlorophyll was slower than in the normal (Table III). Levels of protochlorophyll in etiolated mutants were only 60 to 70% of those in the normal. If these relationships also hold for greening material, the low rate of porphyrin synthesis could be the cause of the lag period in chlorophyll accumulation.

Both retardation of protochlorophyll biosynthesis and instability of chlorophyll once formed could be due to abnormalities in the development of chloroplast structure. Certainly, the plastids of 2- to 3-day mutant seedlings by comparison with normal plastids at the same stage are small, irregular in shape and distorted in structure internally. They appear to be very susceptible to damage by swelling during fixation procedures, whether Rawlins solution or buffered osmium is used, which suggests an increase in permeability of the plastid membrane. The large vesicles in young mutant plastids could have arisen as the result of swelling of double lamellae, and the occasional compartmented vesicles (Figs. 10B, 12) are probably swollen grana. This difference in behaviour of the membranous structures may reflect a qualitative alteration in lipoprotein.

The other possibility, that structural abnormalities in the plastid are the result and not the cause of low pigment levels is less probable. Von Wettstein (1958, 1959) and others have demonstrated that lamellar organisation does not require chlorophyll, though formation of grana does. In the young Gateway mutant chlorophyll levels are evidently high enough to permit formation of at least a few grana at the 2- to 3-day stage (Figs. 10B, 12) and several at the 4-day stage (Fig. 15).

The virescens mutant is able to recover nearly normal levels of chlorophyll within two weeks of germination. This recovery has been found to be accompanied by adjustment towards normality of other cell components. Thus, low carotenoid and nucleotide levels in the young mutant tend to increase with age towards normal levels (Tables I and VIII respectively). Structural integrity and complexity of the older mutant plastid is indistinguishable from the normal (Fig. 18B - 20). Relatively very high acid-soluble nitrogen levels in the young mutant decline rapidly towards the normal during development (Tables IV and V), and the levels of nearly all amino acids follow this pattern (Tables VI and VIII). This is completely opposite to the findings of Seltman (1955) for albino corn seedlings, where free amino acid levels were low in young tissues and increased with age until the death of the seedlings. He attributed the high amino acid content to increased proteolysis brought on by a lack of

carbohydrate in the albino tissues. His argument was based on the fact that when seedlings were grown in darkness the free amino acid levels were the same in the normal as the mutant. Preliminary measurements of nitrogen in etiolated Gateway barley normal and mutant seedlings showed that as in light-grown material, the mutant was higher both in total nitrogen and in acid-soluble nitrogen.

Of special interest are the changes in distribution of nitrogen between particulate and soluble fractions. These changes are summarised in Table IX where the nitrogen contents of the three leaf homogenate fractions (Table IV) are expressed as a percentage of the total nitrogen. In both normal and mutant, the proportion of total nitrogen in the particles increases with age at the expense of acid soluble nitrogen. The change is particularly dramatic in the mutant. This agrees with the findings of de Deken-Grenson (1954) that synthesis of chloroplast protein from acid soluble nitrogenous material occurs during greening and development of chloroplasts. While there may have been turnover of acid-insoluble nitrogen (cytoplasmic protein), there was no net transfer from this fraction to particles during greening (cf. Brawerman and Chargaff, 1959). Recovery in the mutant, therefore, appears to involve the overcoming of a lag in the utilisation of soluble nitrogen for synthesis of particulate nitrogen.

Part of the change in soluble nitrogen is due to a decrease

TABLE IX

DISTRIBUTION OF NITROGEN IN LEAF FRACTIONS AS PERCENTAGE OF TOTAL NITROGEN IN 4-, 6-, and 12-DAY NORMAL AND MUTANT BARLEY SEEDLINGS

Fraction	% of total nitrogen					
	4-Day		6-Day		12-Day	
	N	M	N	M	N	M
Particles	30.7	25.3	34.2	29.2	38.3	38.3
Acid-insoluble	42.8	31.1	44.6	39.1	41.4	34.5
Acid-soluble	26.5	43.6	21.2	31.7	20.3	27.2

in unidentified nitrogen compounds, probably mainly amides (Table VIII). Individual amino acids also decrease (Table VI). Much of this loss in the mutant is attributable to serine, which decreased from 6.7% of the total nitrogen in 4-day leaves to 0.5% in the 12-day leaves (compared with a decrease from 1.3% to 0.3% in normal leaves). This could have accounted for as much as one half the nitrogen increase in the particulate fraction during this time. Although according to Folkes (1959), serine is not a major component of newly formed protein in germinating barley, analysis of protein amino acids involves acid hydrolysis and this procedure was found in the present studies to destroy serine. Serine is also the most common amino acid constituent of certain leaf phospholipids (Stumpf and Bradbeer, 1959). These compounds play an important part as binding elements in lipoprotein membranes. Thus the excessive accumulation of free serine in the young Gateway barley mutant and its subsequent disappearance during greening may be related to the lag in development of chloroplast structure.

In conclusion, it has been shown that disturbances in pigment and nitrogen metabolism in the Gateway barley mutant when young are gradually overcome with age, and that this is accompanied by development of normal structure in the chloroplast. During the lag phase of development of the mutant there is never a total absence of anyone pigment (except, perhaps, chlorophyll _b_), nitrogenous component or structural component, of those examined

which could be suspected as the cause of these abnormalities. The findings are consistent with the interpretation that the abnormalities observed are interdependent and arise out of a disturbance in the quality or an inhibition in the development of a structural chloroplast component during the early stages in seedling growth. Factors difficult to investigate, such as the consequences of changes in plastid permeability, holochrome integrity, etc. may be involved. If this is so, more refined methods are necessary to determine the disturbances.

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APPENDIX

Calculation of formulae for determining concentrations of pigments in acetone solutions.

The concentration of pigments in acetone extracts of leaf material was calculated according to Beer's Law which states that:

$$D = kcd$$

Where D = optical density

k = specific absorption coefficient

c = concentration

d = length of light path through solution

The specific absorption coefficients for chlorophylls a and b and protochlorophyll which were used in calculating formulae are as follows:

Pigment	Wave Length (mu)		
	663	645	623
Chlorophyll a (MacKinney, 1940)	83.0	15.5	14.5
Chlorophyll b (MacKinney, 1940)	5.2	52.5	9.7
Protochlorophyll (Koski and Smith, 1948)	0.2	1.2	35.0

For estimation of chlorophylls a and b in extracts of light-grown leaves (i.e. containing no protochlorophyll) the following simultaneous equations were set up, taking the length of light path as 1 cm.:

$$D_{663} = 83\ C_a + 5.2\ C_b$$

$$D_{645} = 15.5\ C_a + 52.5\ C_b$$

where D_{663} = optical density at 663 mu,

D_{645} = optical density at 645 mu,

C_a = concentration of chlorophyll a in g./l

C_b = concentration of chlorophyll b in g./l

Solving these equations for C_a and C_b:

$$C_a = 0.0123\ D_{663} - 0.00086\ D_{645}$$

$$C_b = 0.0193\ D_{645} - 0.0036\ D_{663}$$

To determine C_a and C_b in mg./g.fr.wt. of tissue, therefore, the following equations were used:

$$C_a = \frac{(12.3\ D_{663} - 0.86\ D_{645})V}{d \times 1000 \times W}$$

$$C_b = \frac{(19.3\ D_{645} - 3.6\ D_{663})V}{d \times 1000 \times W}$$

Where C_a = mg. Chlorophyll a / g.fr.wt.

C_b = mg. Chlorophyll b / g.fr.wt.

V = volume of acetone extract in ml.

d = length of light path in cm.

W = fr.wt. of leaves in g.

For the estimation of chlorophylls a and b and protochlorophyll in etiolated leaves the following simultaneous equations were set up:

$$D_{663} = D_{a_{663}} + \frac{5.2}{52.5} D_{b_{645}} + \frac{0.2}{35} D_{P_{623}}$$

$$D_{645} = \frac{15.5}{83} D_{a_{663}} + D_{b_{645}} + \frac{1.2}{35} D_{P_{623}}$$

$$D_{623} = \frac{14.5}{83} D_{a_{663}} + \frac{9.7}{52.5} D_{b_{645}} + D_{P_{623}}$$

Solving for $D_{a_{663}}$, $D_{b_{645}}$ and $D_{P_{623}}$:

$$D_{a_{663}} = 1.019\, D_{663} - 0.102\, D_{645} - 0.003\, D_{623}$$

$$D_{b_{645}} = -0.181\, D_{663} + 1.025\, D_{645} - 0.034\, D_{623}$$

$$D_{P_{623}} = -0.144\, D_{663} - 0.171\, D_{645} + 1.006\, D_{623}$$

From Beer's Law, $C_a = \dfrac{D_{a_{663}}}{83 \times d}$,

where C_a = concentration of chlorophyll a

83 = sp. abs. coeff. chlorophyll a

d = light path in cm.

The equations used, therefore, in calculating the concentrations of chlorophylls a and b and protochlorophyll were as follows:

$$C_a\ (\text{mg./g.fr.wt.}) = \frac{(1.19\ D_{663} - 0.012\ D_{645} - 0.003\ D_{623})V}{0.083 \times d \times W \times 1000}$$

$$C_b\ (\text{mg./g.fr.wt.}) = \frac{(-0.181\ D_{663} + 1.025\ D_{645} - 0.034\ D_{623})V}{0.0525 \times d \times W \times 1000}$$

$$C_p\ (\text{mg./g.fr.wt.}) = \frac{(-0.144\ D_{663} - 0.171\ D_{645} + 1.006\ D_{623})V}{0.035 \times d \times W \times 1000}$$

where V, d and W are the same as before.

For determinations of carotenoid pigments in acetone extracts of leaves the following equation of von Wettstein (1957a) was used:

$$C_c = 4.695\ D_{440} - 0.268\ (C_a + C_b)$$

where C_c, C_a and C_b are in mg./litre of extract.

TABLE i

DETERMINATION OF RNA WITH ORCINOL AFTER MEJBAUM

(see Methods)

Yeast RNA*(ug./ml.)	Optical Density (at 670mu.)
100	0.103
200	0.210
300	0.334
400	0.440
500	0.562
Average: O.D. 0.110/100 ug.	

* Supplied by Nutritional Biochemicals Corp.

Note: Measurements of the concentration of free nucleotides by this method involves the assumption that the relative concentrations of the purine and pyrimidine nucleotides are the same as in yeast RNA.

FREE AMINO ACID CONCENTRATIONS IN ACID-SOLUBLE FRACTIONS

OF 5-DAY NORMAL AND MUTANT LEAVES

Plants grown in the growth chamber.

Amino acid	um.a.a./g.fr.wt.		$\frac{\text{um.a.a.}}{\text{total um.a.a.}} \times 100$	
	N	M	N	M
Lysine	0.05	0.26	0.7	0.7
Histidine	0.06	0.80	0.8	2.2
Ammonia	0.94	3.67	11.8	10.1
Arginine	0.00	0.14	0.0	0.4
Aspartic acid	0.94	2.77	11.8	7.6
Threonine	0.34	0.91	4.2	2.5
Serine	2.20	20.11	27.5	55.4
Glutamic acid	2.40	3.02	30.0	8.3
Proline	trace	trace	0.0	0.0
Glycine	0.13	0.68	1.7	1.9
Alanine	0.73	1.88	9.1	5.2
Cystine	0.00	0.00	0.0	0.0
Valine	0.12	1.08	1.5	3.0
Methionine	0.00	trace	0.0	0.0
Isoleucine	0.05	0.50	0.6	1.4
Leucine	0.04	0.49	0.5	1.3
Tyrosine	0.00	trace	0.0	0.0
Phenylalanine	trace	trace	0.0	0.0
Total	8.00	36.31		